THE ROLE OF COLLAGEN AND TRANSFORMING GROWTH FACTOR-BETA IN MESOTHELIOMA GROWTH

By Keith Sahan Abayasiriwardana

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Centre for Cardiopulmonary Biochemistry and Respiratory Medicine
University College London and Royal Free Medical School
The Rayne Institute, 5 University Street, London WC1E 6JJ

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Malignant Mesothelioma (MM) is an aggressive tumour of the lung pleura with very poor patient prognosis. MM is unresponsive to all treatment regimes and over the next 30 years an estimated 100,000 fatalities will occur from this disease in Western Europe alone. MM is an extremely fibrous tumour containing abundant amounts of extracellular matrix, including collagen. Cell-matrix interactions are important for tumour progression and MM cells synthesise collagen as well as transforming growth factor-beta (TGF-β), a key regulator of collagen production. Thiaproline, a proline analogue, was used to inhibit collagen production in MM cells *in vitro* and *in vivo* to test the hypothesis that collagen is important for MM growth. Murine MM cells (AC29) were incubated with increasing concentrations of thiaproline with and without TGF-β_1 (1ng/ml) and incorporation of tritiated thymidine and hydroxyproline levels measured as an index of cell proliferation and collagen production (nM hydroxyproline/10^6 cells) respectively. The effect of thiaproline on tumour growth (median weight, mg [range]) was determined in syngeneic mice subcutaneously injected in the flank with 10^6 MM cells. *In vitro*, 10mM thiaproline significantly reduced cell proliferation by over 65% (control 27400±3200; thiaproline 8950±1000 dpm/well, $p<0.001$) and basal and TGF-β_1-induced collagen production by over 50% (control 1.6±0.1; thiaproline 0.7±0.1, $p<0.005$) and 60% (TGF-β_1 3.8±0.2; TGF-β_1+thiaproline 1.3±0.1, $p<0.005$) respectively. At 10 days after injection of cells, 100mg/kg/day thiaproline reduced median tumour weight by over 80% (control 58 [30-105]; thiaproline 10.5 [5-12], $p<0.01$) but no significant difference was seen at 18 days. To summarise, thiaproline inhibited MM cell proliferation, collagen production and delayed tumour growth, suggesting an important role for collagen in MM growth.

MM also secretes TGF-β, which regulates cell growth and collagen production. MM cells produce 30 – 70 times more TGF-β than untransformed normal mesothelial cells. To investigate the role of specific TGF-β isoforms in MM cell proliferation and collagen production and to determine the relative importance in tumour growth, selective neutralising antibodies to TGF-β_1 and TGF-β_2 were used in conjunction with a pan-specific TGF-β antibody which neutralised all TGF-β isoforms. Through the use of the neutralising antibodies it was determined that the predominant isoform

produced by AC29 cells into conditioned medium was TGF-β_2. TGF-β and control antibodies had no effect on AC29 cell proliferation or collagen production *in vitro*. TGF-β and control antibodies (5mg/kg) were injected intraperitoneally into the murine flank model of MM tumour growth at three-day intervals until the end of the experiment, when the tumours were resected and weighed. TGF-β_2 antibody administered *in vivo* significantly decreased median tumour weight compared with PBS and control antibody (PBS control 67.5 [27-114], control antibody 57 [35-193], TGF-β_2 antibody 27 [5-51], $p<0.001$ for both controls). Pan-specific antibody reduced median tumour weight compared to PBS control (PBS control 46.5 [25-109], control antibody 36 [19-107], pan-specific TGF-β antibody 30 [14-66], $p<0.05$ vs. PBS control). Antibodies against TGF-β_1 had no effect on tumour growth. Collagen analysis of the tumours revealed a significantly lower concentration of collagen in the pan-specific antibody treated tumours (PBS control 9.11±0.76, pan-specific TGF-β antibody 4.03±0.68 nM hydroxyproline / mg tumour, $p<0.0001$). Collectively, this thesis has clearly demonstrated that TGF-β-induced collagen production may be an important aspect of MM tumour growth, and inhibition of either collagen production or TGF-β activity can delay MM tumour growth with a resultant decrease in tumour collagen content. A dual approach targeting both collagen and TGF-β production may be of benefit in the treatment of this disease in which the current therapies are inadequate.

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May the blessings of the Triple Gem fall upon you.

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List of Abbreviations

α1(I)	type I collagen alpha chain, and other collagen polypeptide chains accordingly
^{3}H-TdR	tritiated thymidine
A	absorbance
ANOVA	analysis of variance
AP-1	activator protein-1
BAPN	β-aminopropionitrile
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BSA	bovine serum albumin
cdk	cyclin dependent kinase
conc.	concentration
CTL	cytotoxic T-lymphocyte
DAB	3,3'diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
d.p.m.	disintegrations per minute
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EMT	epithelial to mesenchymal transformation
ERK	extracelluar signalling-related kinases
FBS	foetal bovine serum
FGF	fibroblast growth factor
g	gram
x g	gravitational field
GAG	glycosaminoglycans

GDF	growth and differentiation factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HPLC	High Performance Liquid Chromatography
hr	hour
hyp	hydroxyproline
ICTP	carboxyterminal telopeptide of type I collagen
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
IMS	industrial methylated spirit
INF	interferon
i.p	intraperitoneal
JNK	c-jun NH_2 kinase
kb	kilobase
l	litre
LAP	latency associated peptide
LDH	lactate dehydrogenase
LLC	large latent complex
LTBP	latent TGF-β binding protein
m	metre
M	molar
MAPK	mitogen-activated protein kinase
MHC	major histocompatability complex
min	minute
MLEC	mink lung epithelial cell
MM	malignant mesothelioma
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
ODN	antisense oligonucleotide
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PDGF	platelet derived growth factor

PG	proteoglycan
r	correlation coefficient
RER	rough endoplasmic reticulin
R.L.U.	relative light unit
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPM	rat pleural mesothelial cells
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SAPK	stress-activated protein kinases
s.c.	subcutaneous
SCID	severe combined immunodeficient
SEM	standard error of the mean
SF / HGF	scatter factor / hepatocyte growth factor
SLC	small latent complex
T_0	time zero
TβR	TGF-β receptor
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
uv	ultraviolet
v/v	volume per volume
VEGF	vascular endothelial growth factor
w/v	weight per volume

Chapter One

Introduction

* Additionally, repeated exposure to asbestos in certain professions such as carpentry and plumbing in conjunction with the biopersistence of asbestos fibres in the lung also contributes to the rising incidence (Miller *et al.*, 1999).

1.1 Malignant mesothelioma

Lung and pleural cancer are responsible for more deaths than any other malignancy in the developed world and claim the lives of approximately 42,000 in the UK each year (Rintoul and Sethi, 2002). More than 3% of these deaths are caused by malignant mesothelioma (MM), an extremely aggressive tumour of the mesothelial lining of serosal surfaces, occurring primarily in the lung pleura and strongly associated with exposure to asbestos (first implicated by Wagner in 1960). MM is characterised by an extremely fibrous extracellular matrix (ECM) invading the pleural cavity and encasing the lungs, causing shortness of breath and respiratory difficulties.

Patient prognosis is exceptionally poor with MM being almost always fatal. Median survival from diagnosis has been reported to range from 8 to 14 months (Chahinian *et al.* 1982, Antman *et al.* 1988, Ribak and Selikoff 1992). The current treatment is inadequate, with surgery, radiotherapy and chemotherapy showing little improvement in long-term patient survival. The incidence of MM has been increasing rapidly since its first description in the early 1960s. Mathematical modelling suggests that MM related deaths in Britain will continue to rise for the next 20 years, from the current annual total of 1500 to more than 3000 cases per year. This represents as much as 1% of all mortality for men born in the 1940s, one of the worst affected cohorts (Peto *et al.* 1995). A recent report from the British Thoracic Society (2001) showed a 75% increase in MM deaths between 1988 and 1998, and over the next 35 years MM will be responsible for approximately a quarter of a million deaths in Western Europe alone (Peto *et al.* 1999).

There is a long latent period between the initial exposure to asbestos and death, with a study by Yates and colleagues (1997) suggesting a mean of 41 years. It is rare for cases to appear less than 15 years after exposure (Doll and Peto, 1985). The extensive development time of MM accounts for the rising incidence, even 30 to 40 years after the enforcement of strict laws regulating the importation and use of asbestos.

MM is predominantly a male disease; from 1987 to 1991 the recorded death rate from MM in Britain was over 7 times higher in men than women (44 per million for males and 6 per million for females). The discrepancy between the sexes may be due to the higher risk of occupational exposure to asbestos in male dominated employment.

Rates of incidence are higher in professions which involve greater contact with asbestos, such as construction, shipbuilding and carpentry (Peto *et al.*, 1995). Asbestos use in Western Europe remained high until 1980 and large quantities are still used in some European countries today, as well as many developing countries, posing a very real threat to those exposed. Elucidation of the cellular mechanisms involved in MM progression will give greater insight into MM biology and may lead to the development of novel strategies to treat this terminal disease.

1.2 Pathogenesis of malignant mesothelioma

The exact cause of transformation from normal mesothelium to MM has not been determined. However, MM is strongly associated with a previous inhalation of asbestos fibres. Asbestos has been shown to have a number of properties and effects on cells that would be consistent with malignant transformation and tumourigenesis, which are detailed below.

1.2.1 The mesothelium

The mesothelium comprises a monolayer of flattened cells covering serosal cavities and organs, and provides a smooth frictionless surface facilitating movement within the body. Mesothelium covering the internal organs is referred to as the visceral mesothelium, whereas the parietal mesothelium lines the body wall. Embryologically, the mesothelium develops from mesodermal tissue by the gradual differentiation from round or cuboidal cells to elongated flattened cells lining the coelomic cavities (Hesseldahl and Larsen, 1969, Moore, 1982). Mesothelial cells have an epithelial like cobblestone morphology that changes to a more cuboidal appearance upon stimulation (Whitaker *et al.*, 1991). Many studies provide evidence linking ECM components to enhanced tumour progression (examined in section 1.7). Mesothelial cells synthesise ECM-related proteins, including elastin, fibronectin, biglycan, decorin and collagen types I, III and IV (reviewed by Mutsaers 2002). When cultured on bovine corneal endothelial cell ECM (a substrate closely mimicking human mesothelial subcellular basement membrane) mesothelial cells grow more densely and at a faster rate (Niedbala *et al.*, 1986).

1.2.2 Asbestos minerals

Inhalation of asbestos fibres, the collective term for a group of naturally occurring

fibrous minerals, is strongly linked to the malignant transformation of the mesothelium. There are two groups of asbestos minerals, serpentine and amphibole, both of which are capable of causing pulmonary disease. Chrysotile asbestos exists as bundles of 'curly' fibres that break down over time after the leaching of magnesium and silica (Jaurand *et al.*, 1984). Amphibole asbestos is more durable in the lung and has straight rod-like fibres. Amphibole fibres have an iron content of approximately 27%, greater than the 1 – 6 % found in chrysotile fibres mainly as a surface contaminant (Hardy and Aust, 1995).

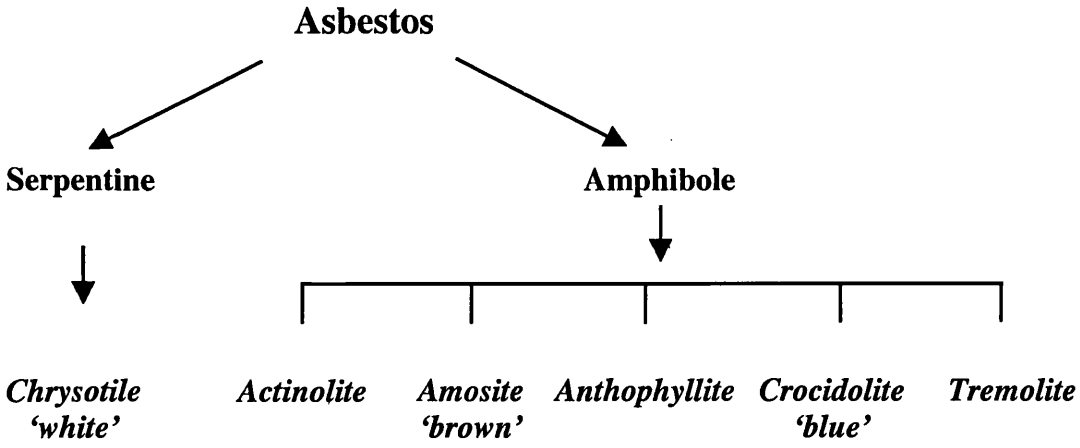


Figure 1.1 Classes of asbestos fibres

Serpentine asbestos accounts for over 95% of the world asbestos consumption (Landrigan, 1998), but is often found in association with amphibole ores. Chrysotile, derived from serpentine minerals, is also referred to as 'white asbestos'. Amphibole minerals contain 5 species of asbestos (figure 1.1): actinolite, amosite ('brown asbestos'), anthophyllite, crocidolite ('blue asbestos') and tremolite. Amphibole fibres are more toxic than chrysotile (Kamp and Weitzman, 1999) with crocidolite being the most pathogenic fibre in the induction of MM in humans (Mossman *et al.*, 1990).

The size and dimensions of fibres have been implicated in the pathogenicity of asbestos. Several animal studies have shown that long thin fibres (>5 μm) are more

* However, these studies involved the direct exposure of fibres to the lung pleura, which is not truly representative of inhaled fibre exposure.

fibrogenic and capable of inducing MM than short fibres (Lemaire, 1985, Platek *et al.*, 1985, Davis *et al.*, 1986). Additionally, longer fibres are more potent inducers of cytotoxicity (Goodglick and Kane, 1990), cell proliferation (Adamson and Bowden, 1987, Woodworth *et al.*, 1983), inflammation (Donaldson *et al.*, 1989) and oxidant production (Mossman *et al.*, 1989).

Interestingly, any fibre, regardless of chemical composition, with dimensions of <0.15 μm in width and >8 μm in length is capable of inducing MM (Stanton *et al*, 1972, 1977, 1981, Davis *et al.*, 1978, Suzuki, 1984). There are over a 100 natural fibrous minerals of these proportions as well as many man-made fibres (Leineweber, 1980). However, inhaled fibre concentration and durability in biological tissue affect the carcinogenicity of the fibres and therefore the only non-asbestos fibre to pose an oncogenic threat to humans is the mineral erionite (Rohl *et al.*, 1982; Baris *et al.*, 1991, 1996). Several regions of Turkey have naturally high deposits of erionite, which is mined and used as a building material. In these villages MM is responsible for more than 50% of the total deaths (Emri *et al.*, 2002).

Numerous animal models support the role of asbestos in tumour induction and demonstrate that administration of asbestos leads to the development of MM (Whitaker *et al.*, 1984, Craighead *et al.*, 1987, Walker *et al.*, 1992, Davis *et al.*, 1992). The exact mechanisms by which asbestos initiates cancer are unknown. Studies in the literature suggest that chromosomal aberrations, cell signalling pathway stimulation and the increased or uncontrolled production of growth factors and their receptors and inflammation may all be important.

1.2.3 Asbestos induces chromosomal abnormalities

Inhaled asbestos fibres accumulate heterogeneously in the lung, concentrating in "black spots" on the parietal pleura (Boutin *et al.*, 1996) demonstrating that asbestos preferentially accumulates near the mesothelial layer with potential carcinogenic effects. Asbestos fibres can induce chromosomal damage in a wide variety of mammalian cells. Hesterberg and Barrett (1985) demonstrated *in vitro* using Syrian hamster embryo cells that asbestos fibres interfere with chromosomal segregation through physical interaction with the mitotic apparatus, thought to result in

aneuploidy and other chromosomal aberrations. Chrysotile and crocidolite have been shown to induce chromosomal damage in rat mesothelial cells, including aneuploidy (Craighead *et al.,* 1987), and translocations and deletions (Libbus and Craighead, 1988). Also, human mesothelial cells exposed to asbestos have an increased number of numerical and structural chromosomal abnormalities (Olofsson and Mark, 1989).

Multiple chromosomal abnormalities are often observed in human MM, perhaps leading to the loss or inactivation of tumour suppressor genes or the activation of oncogenes. Frequent losses occur in many chromosomes such as 1 (Lee *et al,* 1996, Rozet *et al.,* 1998), 6 (Bell *et al.,* 1997, Ribotta *et al.,* 1998), 13 and 14 (Bjorkqvist *et al.,* 1999, De Rienzo *et al.,* 2000), however no common abnormality has been observed in either animal or human MM. A study by Lu *et al.* (1994) showed that over 60% of samples from a series of human MM had an allelic loss at chromosome 3p21, a deletion of which is associated with myeloid leukaemia (Weiser, 2002). Additionally, the DNA mismatch repair gene hMLH1 is also localised to 3p21 and is mutated in hereditary colon cancer (Papadopoulos *et al.,* 1994), suggesting a tumour suppressor role for this region of chromosome 3.

1.2.4 Asbestos generates reactive species

Rich in iron, asbestos fibres catalyse redox reactions that generate the production of reactive oxygen species (ROS), such as superoxide (O_2^-) and the hydroxyl radical ($HO^\bullet$), as well as reactive nitrogen species (RNS; Kamp *et al.,* 1992, Mossman and Churg, 1998, Hardy and Aust, 1995). ROS and RNS can potentially damage proteins, cell membrane lipids, DNA and RNA leading to cellular dysfunction, cytotoxicity and malignant transformation (Kamp and Weitzman, 1999).

1.2.5 Asbestos stimulates cell signalling pathways

The mitogen-activated protein kinase (MAPK) signalling pathway involves a series of phosphorylation events, initiated by the extracellular signalling-related kinases (ERK), c-jun NH_2 kinase (JNK), the stress-activated protein kinases (SAPK) or p38 (Karin, 1995). The MAPK pathway can induce the expression of activator protein-1 (AP-1) transcription factors, such as c-fos and c-jun, which interact with regulatory DNA sequences (Angel and Karin, 1991). Expression of both c-fos and c-jun is

required for the transition of the cell cycle from G_1 to the S phase leading to cell proliferation.

Crocidolite asbestos activates ERK in rat pleural mesothelial (RPM) cells, chelation of the surface iron abrogating this effect (Jimenez *et al.*, 1997). This suggests that iron-catalysed redox reactions are important in asbestos-regulated cell signalling. Asbestos fibres induce phosphorylation of the ERK and MAPK pathways in human mesothelial cells and also directly autophosphorylate the epidermal growth factor receptor (EGFR) leading to the triggering of the ERK cascade (Zanella *et al.*, 1996). Faux *et al.* (2000) have demonstrated that RPM cells have an increased expression of EGFR upon phagocytosing long fibres of crocidolite and erionite. Intense staining for EGFR is seen after 24 hr, which later colocalises with proliferating cell nuclear antigen (PCNA) at 48 hr, suggesting that carcinogenic fibres induce EGFR which initiates cell signalling leading to cell proliferation.

Additionally, RPM cells exposed to asbestos exhibit a dose-dependent increase in c-fos and c-jun mRNA levels and a higher AP-1 DNA binding activity with increased cell proliferation (Heintz *et al.*, 1993, Timblin *et al.*, 1998). Sandhu *et al.* (2000) showed that upon asbestos treatment RPM cells upregulate the proto-oncogenes c-myc, fra-1 and EGFR. These studies suggest that the ability of asbestos to induce key regulators of the cell cycle may be important in the malignant transformation of the mesothelium.

1.2.6 Increased levels of growth factors and receptors are present in mesothelioma

Compared with normal mesothelium, MM produces higher levels of many growth factors and cytokines, which may contribute to malignant growth. Additionally, the uptake of asbestos fibres by macrophages and mesothelial cells results in the release of a variety of cytokines and growth factors which can induce inflammation and further stimulate the release of cytokines (Branchaud *et al.*, 1993, Kane 1992).

Transforming growth factor alpha (TGF-α) is a potent mitogen and inducer of inflammation. TGF-α is a member of the EGF superfamily and is also capable of binding to the EGFR to initiate intracellular cell signalling (Harris *et al.*, 2002).

Elevated levels of TGF-α are present in the serum of asbestosis patients with cancer, in some cases several years before the diagnosis of disease (Partanen *et al.*, 1995). Asbestos transformed rat mesothelial cells express functional EGFR and TGF-α, and neutralising antibodies to TGF-α inhibit the growth of these cells suggesting an autocrine growth loop (Walker *et al.*, 1995), an effect also observed in human MM *in vitro* (Morocz *et al.*, 1994).

Platelet-derived growth factor (PDGF) is a dimeric growth factor consisting of A and B chains, as well as the recently identified C and D chains (Li *et al.*, 2000, Bergsten *et al.*, 2001). Originally isolated from platelets, PDGF is a potent mitogen and chemotactic molecule, and is produced by several cell types including endothelial cells, fibroblasts, macrophages and osteoblasts (Matoskova *et al.*, 1989). There are five identified isoforms of PDGF; PDGF-AA, -AB, -BB, -CC and –DD. Human MM cell lines produce PDGF A and B chains at higher levels than normal mesothelial cells (Gerwin *et al.*, 1987, Versnel *et al.*, 1988, Van der Meeren *et al.*, 1993). Human MM cell lines express PDGF-β receptors (binding PDGF-AB, -BB and –DD) whereas normal mesothelium express predominantly PDGF-α receptors (Versnel *et al.*, 1991), which have an affinity for all PDGF chains apart from D. A significant proportion of human MM were shown to express the PDGF-β receptor whereas non-neoplastic mesothelium did not (Ramael *et al.*, 1992). This has also been demonstrated in rat MM (Walker *et al.*, 1992). PDGF-BB is a chemoattractant for human MM cells (Klominek *et al.*, 1998) and also stimulates MM to produce glycosaminoglycans (Tzanakakis *et al.*, 1995). Transfection of human MM cells with a hammerhead ribozyme that cleaves PDGF B chain mRNA resulted in a decreased expression of PGDF B chain and decreased cell growth *in vitro* and *in vivo* (Dorai *et al.*, 1994) suggesting a role for PDGF B chain in MM growth.

Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis and vascular permeability. Also, VEGF induces endothelial cell proliferation and causes vasodilation through the stimulation of nitric oxide synthase (Yang *et al.*, 1996). VEGF is secreted by most cell types, such as platelets (Pinedo *et al.*, 1998) and fibroblasts (Gentilleti and Fava, 2003), but usually not by endothelial cells themselves. VEGF is at significantly higher levels in the conditioned medium of MM

cell lines compared with normal mesothelial cells (Strizzi *et al.*, 2001). Exogenous VEGF increased MM cell proliferation, and neutralising antibodies to VEGF or its receptors significantly reduced proliferation. VEGF is elevated in malignant pleural effusions (Zebrowski *et al.*, 1999), and has been associated with angiogenesis in human MM (Ohta *et al.*, 1999).

Hepatocyte growth factor / scatter factor (HGF/SF) is produced by mesenchymal and stromal cells, and acts in a paracrine fashion on malignant and non-malignant epithelial derived cells promoting proliferation, motility and invasion (Nishimura *et al.*, 1999, Kuba *et al.*, 2000, Gmyrek *et al.*, 2001). Conditioned media from fibrosarcomatous or mixed phenotype MM cell lines contained HGF/SF which was absent in epithelial MM cell lines (Harvey *et al.*, 1998). Immunoreactivity for HGF/SF has also been observed in tissue sections of human MM samples (Thirkettle *et al.*, 2000), with an association between the expression of HGF/SF and it's receptor (c-met) and increased microvessel density (Tolnay *et al.*, 1998). Klominek *et al.* (1998) observed that human MM cell lines express c-met and that HGF/SF stimulates cell migration and proliferation.

The insulin like growth factor (IGF) family, produced primarily by the liver, consists of IGF-I, -II and proinsulin (Yu *et al.*, 2003). Insulin acts on the liver, muscle and adipose tissue, whereas the IGFs enter the circulation and are important in functions throughout the body (LeRoith, 1997). Both IGFs are essential for embryonic growth, post-natally IGF-I is important in normal cell proliferation, differentiation and postnatal growth, although the physiological role of IGF-II is undetermined (LeRoith, 1997). There is an elevation of IGF–II associated with fibrous tumours of the pleura (Mori *et al.*, 1999, Kerber, 2000). Also, IGF-I increased the production of ECM proteoglycans (shown to be associated with tumourigenesis in section 1.7) by human MM cell lines (Tzanakakis *et al.*, 1996, Syrokou *et al.*, 1999). In a hamster model of MM, temperature-inducible antisense to the IGF-I receptor reduced cell proliferation and the overexpression of the IGF-I receptor resulted in an increased number of tumours *in vivo* (Pass *et al.*, 1996, 1998).

Interleukin 6 (IL-6) is a pleiotropic cytokine that is involved in the regulation of immune responses and haematopoiesis (Tilg *et al.*, 1997). Many different cell types

produce IL-6 in response to infection, trauma or immunological challenge. Depending on the experimental system, IL-6 has been shown to have both pro- and anti-inflammatory effects, although increasing evidence suggests a predominant immunosuppressive role for IL-6 (Tilg *et al.*, 1997). IL-6 is elevated in pleural effusions taken from human MM patients (Monti *et al.*, 1994) and is measurable in the conditioned media of primary MM cell lines in culture (Schmitter *et al.*, 1992, Motoyama *et al.*, 1993). A murine model of MM demonstrated increased levels of IL-6 before macroscopically detectable tumour growth (Bielefeldt-Ohmann *et al.*, 1995). Also, antibodies to IL-6 reduced the disease symptoms (cachexia, abdominal distension and diarrhoea) but not tumour growth. Interferon α treatment reduced IL-6 at both the mRNA and protein level and increased the number of tumour infiltrating lymphocytes and macrophages, suggesting that IL-6 is an immunosuppressor in MM leading to enhanced tumour growth.

Transforming growth factor-beta (TGF-β) is a multitalented cytokine, which has crucial developmental and homeostatic functions in an array of tissue types (section 1.3). There is evidence that TGF-β levels are raised in MM. Kuwahara *et al.* (2001) have reported that rat MM cells produce 30 – 70 times more TGF-β than normal rat mesothelial cells. Also, Madea and co-workers (1994) investigated the levels of TGF-β in pleural effusions taken from MM and primary lung cancer patients, and found that TGF-β levels in MM were 3 – 6 times higher than in primary lung cancer. Fitzpatrick *et al.* (1994) have shown that MM cells produce both TGF-β_1 and -β_2 and that their inhibition with inducible antisense RNA reduced the anchorage-independent growth of MM cells *in vitro* and the tumourigenicity of MM cells *in vivo*. Additionally, Marzo *et al.* (1997) demonstrated that antisense oligonucleotides specific to TGF-β_2 inhibit the *in vitro* and *in vivo* growth of MM. Collectively, these studies provide very strong evidence suggesting a key role for TGF-β in MM tumourigenesis.

In summary, the pathogenesis of MM is unknown. However, asbestos has been shown to induce chromosomal mutations and to stimulate cell signalling pathways. Additionally, aberrations in signalling pathways, growth factors and their receptors are observed in MM. One such growth factor, TGF-β, is found at extremely elevated levels in MM. Furthermore, TGF-β is the most potent stimulator of collagen

A role for Simian Virus 40 in the development of MM has also been suggested, however this is controversial (literature reviewed by Carbone *et al.*, 2003).

production characterised to date. These observations suggest that the elevated level of TGF-β associated with MM may be contributing to the extremely fibrous nature of this tumour with characteristically dense ECM. The role of TGF-β in MM, and other tumour settings, is reviewed in the following section.

1.3 Transforming growth factor-beta and tumourigenesis

The TGF-β superfamily consists of a large number of structurally related polypeptides. Members of this superfamily have a wide and varied role in the development, homeostasis and repair of virtually all tissues in organisms (Massague, 1998). The TGF-β superfamily contains several subfamilies, including the bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), activins and the TGF-βs.

The most widely studied member of the superfamily is TGF-β, of which there are 5 known isoforms (TGF-$\beta_{1\text{-}5}$). Three mammalian TGF-β isoforms exist, each encoded by separate distinct genes, and are expressed in a tissue specific fashion (Pasche, 2001). There is high homology amongst the isoforms and they are tightly conserved between species indicating a critical biological function. TGF-β is a multifunctional cytokine regulating many key processes such as cell proliferation, differentiation, adhesion, motility, mobility, apoptosis, immune response, angiogenesis and ECM deposition (Hsu *et al.,* 2002). Overproduction of TGF-β is known to lead to disease states where the accumulation of ECM is exaggerated. Due to the regulatory role of TGF-β and the recent elucidation of the TGF-β signalling pathway (section 1.3.1) there has been a great deal of interest in the role of TGF-β in tumour progression. Elevated levels of TGF-β protein production have been found in association with many different tumour types, such as brain tumours (Chen *et al.,* 1997), lung carcinomas (Kong *et al.,* 1996), gastrointestinal malignancies (Saito *et al.*, 1999, Ebert *et al.,* 2000) as well as MM (Kuwahara *et al.*, 2001, Madea *et al.,* 1994). However, the role of TGF-β in promoting MM tumour growth has not been fully elucidated.

TGF-β is secreted from the cell in a latent dimeric complex consisting of the C-terminal mature TGF-β and an attached N-terminal pro-domain, LAP (latency

associated peptide), bound together by disulphide bonds (Gentry *et al.,* 1988). This complex is referred to as the small latent complex (SLC, figure 1.2). The interaction between TGF-β and LAP results in the inactivation of the biological activity of TGF-β; β1-LAP can inactivate all of the mammalian TGF-β isoforms with no isoform specific differences (Miller *et al.,* 1992).

The SLC can also bind to latent TGF-β binding protein (LTBP); LTBP forms a covalent bond with the LAP portion of the SLC (Saharinen *et al.,* 1999). When latent TGF-β associates with LTBP the whole complex is known as the large latent complex (LLC, figure 1.2). The LTBP portion of the LLC has been demonstrated to facilitate the binding of latent TGF- β_1 to the ECM (Taipale *et al.,* 1994), and LTBP contains three domains shown to be capable of binding to human dermal or lung fibroblast matrices (Unsold *et al.,* 2001). It has been found that most cell cultures secrete TGF-β as part of the LLC (Koli *et al.,* 2001).

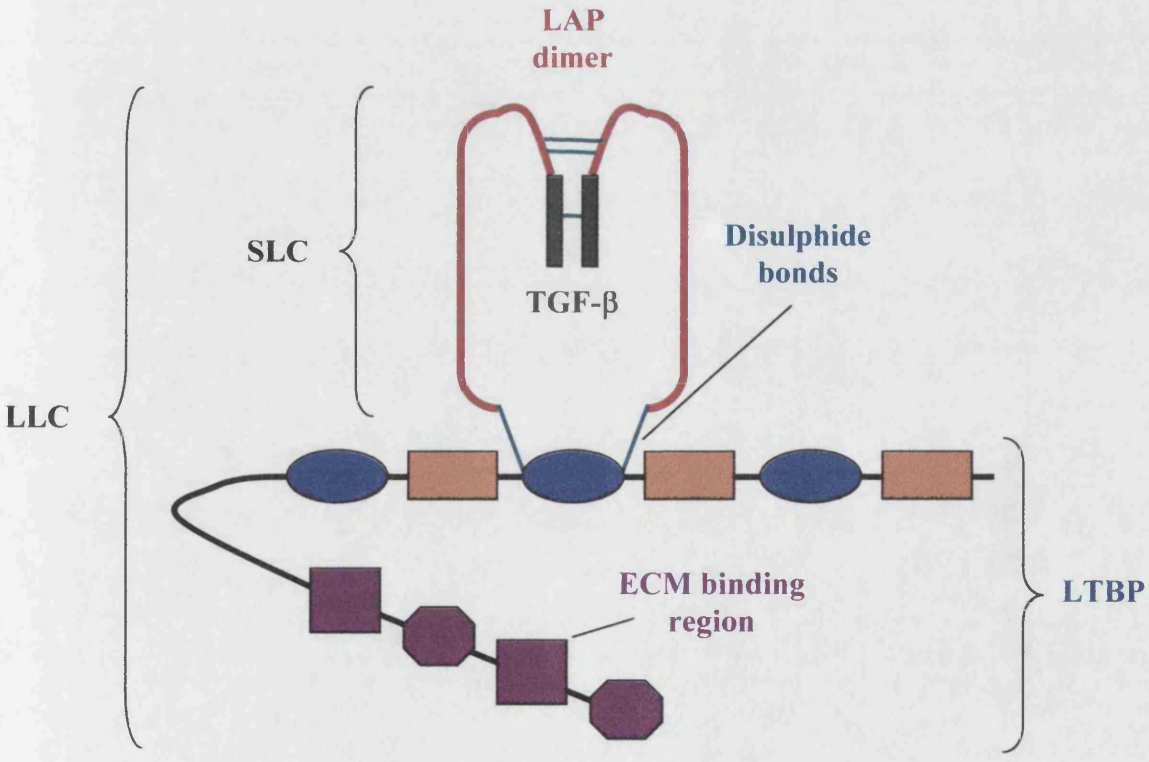


Figure 1.2 Schematic representation of the large latent TGF-β complex. The small latent complex (SLC) contains the C-terminal mature TGF-β and it's N-terminal pro-domain, LAP (latency associated peptide). This complex associates with latent TGF-β binding protein (LTBP) via disulphide bonds to form the large latent complex (LLC). Modified from Annes *et al.* (2003).

LTBP is a family of four separate genes which give rise to four main isoforms of LTBP (1 – 4), although splice variants do exist (Unsold *et al.*, 2001). LTBP-1 is found with fibronectin and fibrillin-rich matrices (Taipale *et al.*, 1996, Isogai *et al.*, 2003) and LTBP-2 is associated with microfibrillar structures and fibronectin-rich fibres (Koli *et al.*, 2001). LTBP-3 is the smallest LTBP and can only be secreted from cells in association with TGF-β_1 (Chen *et al.*, 2002, Penttinen *et al.*, 2002), and may be important in the secretion and targeting of TGF-β_1. The recently characterised LTBP-4 has also been shown to be ECM-bound and to be susceptible to proteolytic release by plasmin (Saharinen *et al.*, 2002).

Matrix bound TGF-β is released from the LLC by multiple proteinases from the same family of serine proteases, including plasmin, cathepsin D, mast cell chymase and leukocyte elastase (Lyons *et al.*, 1990, Taipale *et al.*, 1992, 1995). It is believed that cleavage of LAP from the SLC to yield bioactive TGF-β also occurs at the same time (Koli *et al.*, 2001). LAP can also be cleaved from the SLC by the $\alpha_v\beta_6$ integrin. It has been demonstrated that $\alpha_v\beta_6$ integrin induces the activation of SLC associated TGF-β and that $\alpha_v\beta_6$ null mice exhibited exaggerated inflammation and are protected from bleomycin induced fibrosis, consistent with a decrease in active TGF-β (Munger *et al.*, 1999).

1.3.1 TGF-β mediated intracellular signalling

The predominant TGF-β signalling cascade, the Smad pathway, is outlined in figure 1.3. TGF-β binds to the TGF-β cell surface receptor II (TβRII), part of a family of transmembrane glycoprotein receptors with large extracellular domains and a cytoplasmic serine/threonine kinase domain. The kinase activity of TβRII and subsequent association with TβRI are essential for TGF-β signalling (Wrana *et al.*, 1992). Once bound, TGF-β recruits TβRI into the complex to be transphosphorylated by TβRII allowing the phosphorylation of cytoplasmic substrates to proceed (Wrana *et al.*, 1994). A third group of TGF-β receptors are also expressed on the cell surface, TβRIII, although they are not directly involved in TGF-β signalling. TβRIII has two related members, endoglin and betaglycan, which are also transmembrane glycoproteins with large extracellular domains, but have a small intracellular portion

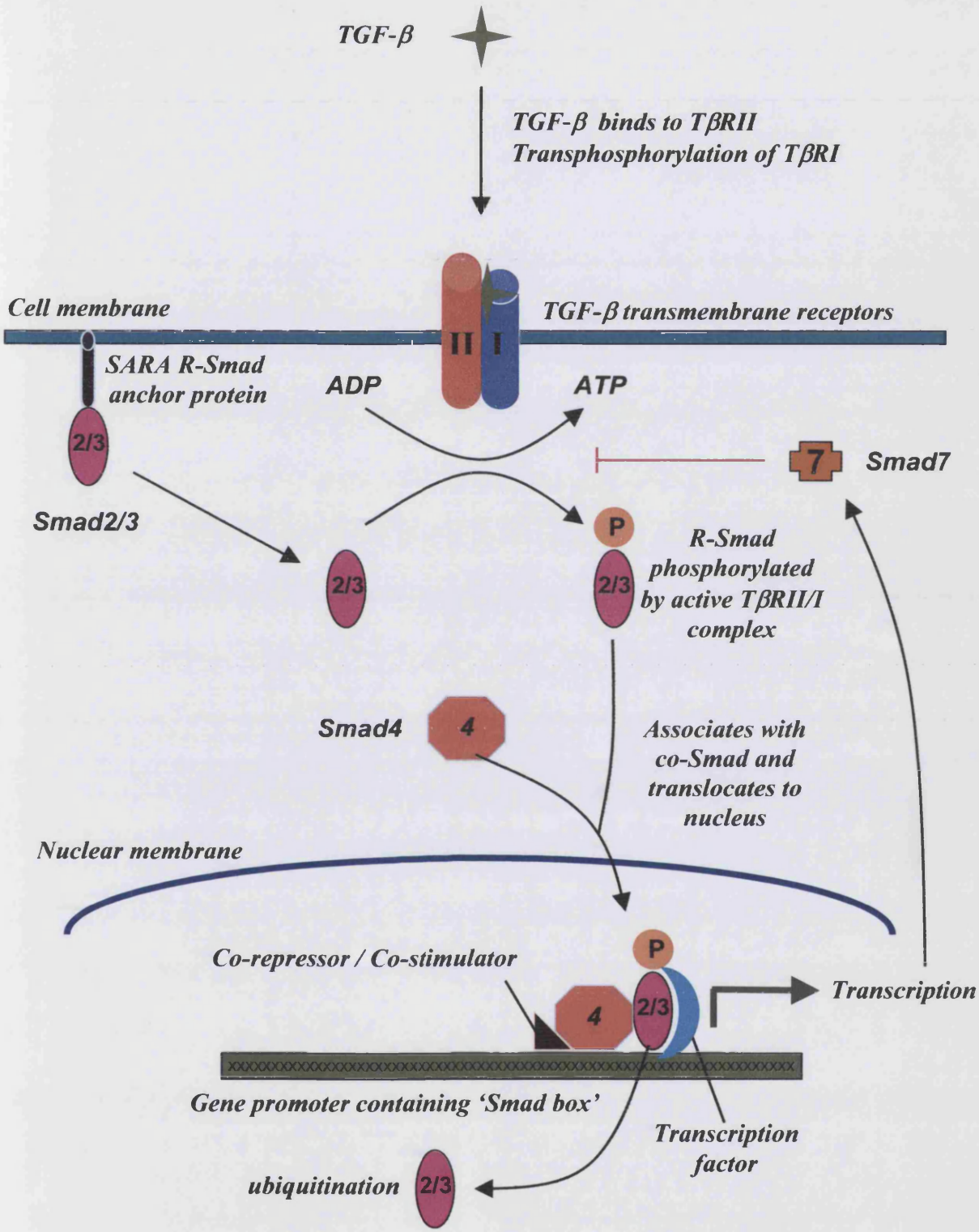


Figure 1.3 TGF-β Smad mediated intracellular signal transduction. Activated Smad complexes translocate to the nucleus and associate with transcription factors, co-stimulators and repressors to drive transcription. TGF-β induces Smad7 production, which inhibits R-Smad phosphorylation and prevents TGF-β signalling. Smad proteins are marked for degradation via proteosomes by ubiquitination.

that has no kinase domain and no identifiable signalling function (Lopez-Casillas *et al.*, 1993). TβRIII is considered to be an accessory receptor regulating the interaction of TGF-β with the functional signalling receptors (Wang *et al.*, 1991). However, TβRIII null mice develop lethal heart and liver defects *in utero* (Stenvers *et al.*, 2003). Furthermore, cells cultured from the embryonic TβRIII knockout mice exhibit reduced sensitivity to TGF-β_2-induced growth inhibition and reporter gene activation, effects not observed with the other TGF-β isoforms. These results suggest an important role for TβRIII in mediating TGF-β_2 function.

The Smad family of signalling molecules mediates BMP / TGF-β intracellular signalling from the activated receptor complex. The mammalian family of Smads consists of 8 structurally related proteins, Smad1 to Smad8. Based on function, the Smads can be divided into three groups (Hill, 1999), receptor-regulated Smads (R-Smads), the common mediator Smad (co-Smad) and inhibitory Smads (anti-Smads). BMP signalling is mediated by R-Smads 1, 5 and 8 and anti-Smad6 whereas TGF-β signalling is mediated by R-Smads 2 and 3 and anti-Smad7 (Piek and Roberts, 2001). Both pathways use Smad4 as the co-Smad.

During TGF-β signalling, the intracellular portion of the TβRII/I activated complex associates with R-Smads, which become phosphorylated themselves (Wrana *et al.*, 1994). The chaperone protein SARA (Smad Anchor for Receptor Activation) localises co-Smads to the cell membrane and recruits them to the activated TβRII/I complex (Wu *et al.*, 2000). After dissociation from the receptor the activated R-Smad associates with the co-Smad, Smad4, and this complex translocates to the nucleus and initiates transcription (Nakao *et al.*, 1997a). Anti-Smads can form a stable association with the activated TβRI thereby blocking the association and subsequent activation of R-Smads (Nakao *et al.*, 1997b). Smad7 mRNA remains localised with the nuclear membrane, but is translocated to the cytoplasm upon TGF-β_1 stimulation (Itoh *et al.*, 1998). Smad7 mRNA is highly induced by TGF-β_1, stimulated by Smad3/4 binding to the Smad7 gene promoter and initiating transcription (Nagarajan *et al.*, 1999). This demonstrates that Smad7 is part of a negative feedback loop regulating TGF-β responses.

Upon nuclear transportation, the phosphorylated Smad complex binds to the 'Smad box' within a gene promoter, a GTCT rich motif that drives transcription (Johnson *et al.*, 1999). The Smad complex can interact with an array of transcription factors and transcriptional co-stimulators or co-repressors, the balance of which allows tight control of TGF-β-induced transcription. The transcription factor CREB binding protein (CBP) / p300 assists Smad-induced transcription. Studies have demonstrated that inhibition of CBP function decreased the level of TGF-β-induced transcription (Feng *et al.*, 1998). Smads can also bind directly to transcription factors from the Jun family of AP-1 transcription factors (Liberati *et al.*, 1999). The oncoprotein Evi-1 interacts with Smad3 and inhibits Smad3 and Smad3/4 transcriptional activation (Kurokawa *et al.*, 1998). The proto-oncoprotein Ski prevents association of Smad2/3 complex with CBP / p300 thereby preventing TGF-β transcriptional activation (Akiyoshi *et al.*, 1999). The proto-oncoprotein SnoN also reduces TGF-β transcriptional activity. Overexpression of SnoN in human foetal kidney cells has been shown to repress Smad3 transcriptional activity (Sun *et al.*, 1999). Phosphorylated R-Smads are ubiquitinated in multiple sites in response to TGF-β, and the nuclear accumulation of ubiquitinated Smads results in their degradation by proteasomes (Lo and Massague, 1999).

The Smad pathway is recognised as the predominant TGF-β mediated intracellular signalling cascade, but TGF-β can also signal via the MAPK and ERK pathways (reviewed by Mulder, 2000). There is likely to be cross talk between the different signalling pathways allowing further cellular control of TGF-β signal transduction. Smad signalling and MAPK/JNK signalling converges at AP1-binding promoter sites with a co-operation between the two signalling pathways to mediate TGF-β-induced transcription (Zhang *et al.*, 1998). TGF-β induces an association of c-Jun and c-Fos (JNK pathway intermediates) with Smad3 (Engel *et al.*, 1999). Both Smad and JNK pathways may be required for the full signalling response as stimulation of the JNK pathway alone resulted in a lower transcriptional activity when compared to activation of both pathways together (Engel *et al.*, 1999).

1.3.2 TGF-β function

TGF-β has a multifunctional physiological role initially playing a vital role in specifying developmental fate. TGF-β isoforms are expressed differentially during development and the differing phenotypes of the TGF-β_1, -β_2 and -β_3 null mice provide evidence for distinct TGF-β isoform roles *in vivo* (Shi *et al.*, 1999). Approximately three weeks after birth, TGF-β_1 null mice develop a progressive wasting disease leading to death, and examination reveals a massive autoimmune inflammation affecting multiple organs such as the heart, lungs, and salivary glands (Shull *et al.*, 1992). TGF-β_1 null SCID mice exhibited no inflammation and survived well into adulthood (Diebold *et al.*, 1995). This suggests the importance of TGF-β_1 in suppressing inflammation. Also, when treated with carcinogens, TGF-β_1 heterozygous mice have an increased development of lung and liver cancers, implicating TGF-β_1 as a tumour suppressor (Tang *et al.*, 1998).

Two-thirds of TGF-β_2 null mice die prenatally and the remaining third are born with impaired oxygen perfusion and die shortly after birth with cardiovascular, skeletal and craniofacial defects (Sanford *et al.*, 1997). There is little phenotypic overlap with the other TGF-β isoform null mice suggesting numerous non-compensated functions for TGF-β_2. Targeted disruption of murine TGF-β_3 results in death within 24 hr of birth due to abnormal pulmonary development, and the pups exhibit a cleft palate (Kaartinen *et al.*, 1995). Disruption of the murine TβRII gene causes early embryonic lethality and hyperplasia of T-cells resulting in the expansion of lymph nodes and spleen (Oshima *et al.*, 1996, Lucas *et al.*, 2000).

TGF-β_1 is the most well characterised isoform. Experimentally, isoform specific effects of TGF-β have not been conclusively established, although the differing phenotypes of the TGF-β knockout animals suggests that there may be non-overlapping functions of the different TGF-β isoforms. Shah *et al.* (1995) have shown that the neutralisation of endogenous TGF-β_1 and -β_2 reduced scarring in a rat model of wound healing, an effect also achieved by the application of exogenous TGF-β_3. This suggests that TGF-β_1 and -β_2 have a different role to TGF-β_3 in wound healing.

Cell transformation

In culture TGF-β can initiate the transformation of epithelial cells into fibroblastoid cells, a process termed epithelial to mesenchymal transformation (EMT) (Miettinen *et al.,* 1994). EMT is essential to the malignant transformation of early stage tumour cells into fully malignant and invasive carcinomas, the acquisition of fibroblastoid features enhancing stromal and vascular invasion (Oft *et al.,* 1996). Cui *et al.* (1996) demonstrated in a TGF-β_1 over-expressing murine model of skin carcinogenesis that TGF-β_1 initially was a tumour suppressor inhibiting malignant transformation. However, in those animals that did develop skin tumours the malignant conversion rate was greatly increased, with a much higher incidence of the more malignant spindle cell carcinomas. Further evidence for the importance of TGF-β in EMT has been demonstrated by Portella *et al.* (1998). Keratinocytes cultured with TGF-β_1 were transformed to a malignant metastatic keratinocyte cell line. When cloned and injected subcutaneously into nude mice the cell lines spontaneously converted to a fibroblastoid phenotype. However, those clones that had been stably transfected with a dominant negative TβRII were unable to undergo this mesenchymal switch *in vivo*, demonstrating the importance of an intact TGF-β signalling pathway in EMT. Additionally, the induction of Raf, a serine/threonine protein kinase which activates the ERK/MAPK pathways, increased TGF-β production in Madin-Darby Canine Kidney (MDCK) epithelial cells (Lehmann *et al.*, 2000). The increase in TGF-β production resulted in EMT of the MDCK cells, whilst also inhibiting TGF-β-induced apoptosis. The invasive behaviour of the MDCK cells through collagen type I gels was also enhanced. Although untested in MM, these studies demonstrate that TGF-β may advance tumour progression by the stimulation of EMT, whilst suppressing apoptosis and increasing cell migration.

Cell proliferation

TGF-β has varying effects on cell proliferation, depending on both the cell type and the concentration of the growth factor. All three mammalian TGF-β isoforms inhibit the proliferation of epithelial and endothelial cells (Cheifetz *et al.,* 1990). TGF-β_1 is a potent inhibitor of epithelial cell proliferation, reducing the expression of cyclin dependent kinase (cdk) genes, such as c-myc and KC, which drive the cell cycle past checkpoints (Howe *et al.,* 1991, Coffey *et al.,* 1988). TGF-β also exerts growth

inhibitory effects by inducing the expression of cell cycle progression suppressor proteins such as cdk inhibitor $p27^{Kip1}$ (Polyak *et al.*, 1994).

TGF-β has been demonstrated to have concentration dependant effects on several cell lines. For example, TGF-β stimulated fibroblast (Kimura *et al.*, 1989) and smooth muscle cell (Battegay *et al*, 1990) proliferation at low concentrations, but inhibited proliferation at higher concentrations. Additionally, the culture of human subconjunctival fibroblasts with an optimal concentration of TGF-β (10^{-6} mM) increased proliferation and migration, but concentrations above or below this peak decreased proliferation and migration (Cordeiro *et al.*, 2000). These observations demonstrate that TGF-β has biphasic effects on cell proliferation.

TGF-β_1 induces the production of prostaglandin E_2 (PGE_2) in human fibroblasts, which inhibits cell proliferation and collagen production (Keerthisingam *et al*, 2001). TGF-β-induced anti-proliferative effects have also been observed in a human breast cancer cell line (Fenig *et al.*, 2001). In this study, endogenous TGF-β_1 stimulated the production of bFGF, which reduced the proliferation of the cancer cell line.

The stimulatory effect of TGF-β on murine cell proliferation in culture has been attributed to induction of autocrine PDGF. This in turn causes upregulation of c-fos and c-myc, both of which are important for the G_1 to S phase transition of the cell cycle (Leof *et al.*, 1986). TGF-β_1-induced proliferation of human foreskin fibroblasts in culture was blocked with PDGF antibodies (Soma and Grotendorst, 1989). Furthermore, the subcutaneous injection of TGF-β_1 into rat skin resulted in an upregulation of PDGF expression *in vivo*, further supporting the role of PDGF in TGF-β-induced proliferation. Additionally, in human fibroblasts TGF-β_1 has been demonstrated to induce the production of CTGF which is a potent fibroblast mitogen (Howell *et al.*, 2001).

Interestingly, studies on rat and murine MM have shown that neither endogenous nor exogenous TGF-β altered MM cell proliferation compared to the controls (Fitzpatrick *et al.*, 1994, Kuwahara *et al.*, 2001). This suggests that the pro-tumourigenic effects

of TGF-β in MM act independently of proliferation, perhaps by suppressing the immune response, or enhancing angiogenesis and ECM production.

Immune suppression

Evasion from the host immune system allows the rapid unchecked proliferation of malignant growths that would otherwise be targeted by inflammatory cells. The most dramatic example of the importance of TGF-β in immune suppression is evidenced in the TGF-β_1 null mice which die shortly after birth from autoimmune disease (Shull *et al.*, 1992). TGF-β is a potent inhibitor of cytotoxic T-lymphocyte (CTL) generation from mixed lymphocyte cultures and inhibits their proliferation (Ranges *et al.*, 1987). The overexpression of TGF-β_1 in an UV-induced fibrosarcoma murine tumour cell line by cDNA stable transfection resulted in enhanced tumourigenicity due to evasion of host T-cell mediated tumour cytotoxicity (Torre-Amione *et al.*, 1990). The expression of antisense oligonucleotides against TGF-β_1 and TGF-β_2 in a murine MM cell line reduced *in vivo* tumourigenicity and also increased the number of tumour infiltrating CTLs (Fitzpatrick *et al.*, 1994). Evidence suggests that TGF-β may have biphasic effects on CTLs, at low concentrations stimulating proliferation (Kondo *et al.*, 1993) whilst at higher concentrations inhibiting proliferation (Beck *et al.*, 2001). These observations suggest that the high levels of TGF-β observed in MM are immunosuppressive, promoting tumour growth. However, this section demonstrates that TGF-β has many protumourigenic properties, and other TGF-β related effects aside from immune suppression might be promoting MM tumour growth.

Apoptosis

TGF-β is a potent inducer of cell death, the initiation of apoptosis in cells of the immune system contributing to its immunosuppressive properties. Although no evidence exists for TGF-β-induced apoptosis in MM, this has been observed in other tumours. TGF-β induces growth arrest and apoptosis in human lymphocytes (Chaouchi *et al.*, 1995, Lomo *et al.*, 1995). The induction of apoptosis in a B-cell lymphoma cell line has been demonstrated to be Smad pathway dependent. Retroviral transfection with dominant negative Smad2 or Smad3 blocked TGF-β mediated apoptosis and also retroviral overexpression of the inhibitory Smad7 protected against apoptosis in a dose-dependent fashion (Patil *et al.*, 2000). This has

also been demonstrated in hepatocytes, myeloid and epithelial cells (Yamamura *et al.*, 2000). The balance of pro- and anti-apoptotic factors determines cellular fate (Schuster and Krieglstein, 2002). Saltzman *et al* (2002) demonstrated in a B-cell lymphoma cell line that TGF-β decreased the expression of the anti-apoptotic protein BCL-X_L, a member of the BCL-2 family, whilst stimulating the apoptotic caspase pathway.

Several tumour cell lines respond to TGF-β-induced apoptosis. The expression of a truncated TβRII in a breast carcinoma cell line resulted in resistance to TNF-α-induced apoptosis, with molecular analysis revealing an alteration in the expression of BCL-2 protein expression (Tobin *et al.*, 2001). The transfection of a murine glioma cell line with TGF-β_1 enhanced the level of TNF-α mediated apoptosis *in vitro* and improved survival rates in an *in vivo* murine glioma model (Ashley *et al.*, 1998). Treatment of glioma and trigeminal neurinoma cells with TGF-β_1 *in vitro* resulted in an inhibition of cell proliferation followed by cell rounding and detachment leading to apoptosis (Marushige and Marushige, 1994).

Although a clearly defined pro-apoptotic role for TGF-β is evident in the literature, Lei *et al* (2002) observed in a breast cancer cell line that inhibition of TGF-β by expression of soluble TβRIII resulted in an increase in apoptosis, suggesting that in this case TGF-β inhibits apoptosis. The role of TGF-β suppressed apoptosis in MM is unknown, although MM produces elevated levels of TGF-β in comparison to normal mesothelial cells and other primary tumours (Fitzpatrick *et al.*, 1994, Maeda *et al.*, 1994). It is possible that the high levels of TGF-β produced by MM may inhibit apoptosis occurring in the tumour cells and thereby enhance tumour growth.

Angiogenesis

Angiogenesis is essential for malignant growth beyond 1 – 2 mm in diameter (Edwards *et al.*, 2001). Tumour-induced neovascularisation ensures an adequate supply of nutrients to the growing tumour and also increases the rate of metastasis, where cells detached from the primary tumour are able to reach the blood system and travel to distant sites. TGF-β stimulates angiogenesis by a combination of direct and indirect effects. For example, the angiogenic factor VEGF is induced by TGF-β

(Pertovaara *et al.*, 1994). Also, the increase in ECM production by TGF-β may be important in vascularisation, as TGF-β treated endothelial cells grown in collagen gels were stimulated to form tube-like vessel structures (Madri *et al.*, 1988). TGF-β can also recruit inflammatory cells, connective tissue cells and epithelial cells that express VEGF in response to TGF-β (Roberts and Sporn, 1989). VEGF can directly stimulate the migration and invasion of endothelial cells in culture through a collagen matrix (Vernon and Sage, 1998). Neutralising antibodies to TGF-β reduced the number of vessels observed histologically within human prostate cancer solid tumours in a subcutaneous murine flank model (Tuxhorn *et al.*, 2002). Elevated levels of TGF-β have been shown to be significantly correlated to increased angiogenesis and worse patient prognosis in human colorectal carcinomas (Xiong *et al.*, 2002) and to increased microvessel density and depth of tumour invasion in human gastric carcinomas (Choi *et al.*, 1997). The importance of TGF-β mediated angiogenesis to MM growth is unknown.

ECM elaboration

The ECM is a prerequisite for malignant tumour growth (Dvorak, 1986). There is a wealth of data in the literature suggesting the importance of ECM components in malignant progression (reviewed in section 1.7). TGF-β has a major role in the regulation of ECM formation, degradation and remodelling (Kingsley, 1994). Also, TGF-β strongly stimulates the expression of many different matrix proteins, such as fibronectin, vitronectin, tenascin, elastin, proteoglycans and collagen (Saharinen *et al.*, 1999). All isoforms of TGF-β are potent stimulators of collagen production *in vitro* (Coker *et al.* 1997). Exogenous TGF-β has been shown to increase the mRNA stability of ECM components collagen, fibronectin and thrombospondin in confluent cultures of murine 3T3 cells, which results in a higher yield of protein product (Penttinen *et al.*, 1988).

Smads 3 and 4 have been demonstrated to be crucial for the TGF-β mediated transcription of human collagen genes (Vindevoghel *et al.*, 1998, Chen *et al.*, 2000**a**). In the case of the α2(I) collagen gene a Smad3 DNA binding site within the gene promoter was identified, which when disrupted led to a decrease in α2(I) collagen transcription (Chen *et al.*, 2000**a**). Also, Vindevoghel *et al.* (1998) have demonstrated

that TGF-β induced transcription of the human type VII collagen gene (COL7A1) promoter is dependent upon Smad4 in breast carcinoma cells. Additionally, Chen *et al.* (1999, 2000a) have shown that the interaction of Smad3 with the human α2(I) procollagen gene promoter is required for the full TGF-β transcriptional response in fibroblasts.

A study by Nakao *et al.* (1999) provides further evidence demonstrating TGF-β-induced collagen production. It was shown that adenovirus mediated expression of a Smad7 transgene (preventing TGF-β signalling) significantly suppressed bleomycin-induced fibrotic changes in mice, with a reduction in type I procollagen mRNA production, hydroxyproline content and Smad2 phosphorylation. Interestingly, Smad7 expression did not affect the numbers of macrophages, lymphocytes or neutrophils in the BALF.

TGF-β also increases the turnover of the ECM by repressing the synthesis of TIMPs (potentially increasing MMP-induced degradation) and increasing the activity of lysyl oxidase, an enzyme vital for the stability of collagen which catalyses cross-link formation (Feres-Filho *et al.*, 1995). TGF-β decreases the proteolytic activity of cells by increasing the expression and secretion of several proteases and protease regulators such as matrix metalloproteinases, plasmin, plasmin activators and plasmin activator inhibitor (Massague, 1998).

TGF-β itself can be associated with the ECM in a latent form through LTBP (Taipale *et al.*, 1994) and can be released by matrix degrading proteinases (Lyons *et al.*, 1990, Sato *et al.*,1990). Interestingly, there appears to be a feedback mechanism whereby the formation of ECM decreases TGF-β_1 gene expression (Streuli *et al.*, 1993), thereby limiting further ECM deposition. However, MM is phenotypically extremely fibrous with a matrix rich in collagen, and also produces high levels of TGF-β, suggesting that this feedback loop is not present. The combination of elevated TGF-β and ECM present in MM provides a large reservoir of TGF-β within the tumour vicinity that may exert pro-tumourigenic effects. Possible mechanisms by which TGF-β could promote tumour growth are detailed below.

1.3.3 Mechanisms for the promotion of tumourigenesis by TGF-β

As described above, TGF-β is a potent inhibitor of cell proliferation and can also induce apoptosis. However, in some carcinomas a switch occurs during the malignant process and the tumour suppressor functions of TGF-β are blocked. The growth inhibitory and apoptotic effects are lost whilst other TGF-β responses that are beneficial to tumour growth, such as the promotion of angiogenesis and ECM production, remain intact (Akhurst, 2002). The role of TGF-β_1 on early and late malignant growth was investigated in a model of skin carcinoma using TGF-β_1 overexpressing transgenic mice (Cui *et al.*, 1996). During the early stages of malignancy TGF-β_1 acted as a tumour suppressor inhibiting malignant conversion. However, overexpression of TGF-β_1 in established tumours resulted in a more invasive phenotype. This study demonstrated a bimodal action of TGF-β_1 in melanoma tumour spread, as during early tumour growth TGF-β_1 was growth inhibitory but stimulatory at later stages.

How does this switch from growth inhibition to tumour promotion in response to TGF-β occur? High expression levels of TβRII are important for inhibition of cell proliferation, whereas the production of tumour promoting ECM components are retained at low TβRII levels, suggesting that a higher intensity of TGF-β signalling is required for proliferation modulation responses (Chen *et al.*, 1993, Cui *et al.*, 1995). Also, Smad2 and Smad3 knockout mice have different phenotypes suggesting that they exert different roles in TGF-β signal transduction (Waldrip *et al.*, 1998, Datto *et al.*, 1999). Thus, the balance of activated TβRII/I and Smad 2/3 may be important in determining downstream signalling targets (Piek and Roberts, 2001). Therefore, mutations or alterations in the expression of TGF-β signalling pathway components may result in a change in TGF-β response, such as loss of the tumour suppressing inhibition of cell growth whilst retaining responses contributing to malignant growth.

TGF-β and related signalling pathway components are altered in a host of malignancies. Elevated production of TGF-β resulting in an increased tumour growth rate *in vivo* has been demonstrated in breast carcinoma (Tobin *et al.*, 2002), prostate cancer (Steiner and Barrack, 1992) and sarcoma (Chang *et al*, 1993). Also, as

previously mentioned, MM produces highly elevated levels of TGF-β in comparison to normal mesothelial cells and other tumours (Kuwahara *et al.*, 2001, Madea *et al.*, 1994), although the role of TGF-β in MM growth is not fully understood.

Resistance to the tumour suppressor functions of TGF-β in malignant growth occurs through a variety of mechanisms. Mutations in the TGF-β receptors would alter downstream signalling. Mutations in the TβRII gene occur in colon cancers (Grady *et al.*, 1999), gastric tumours (Yang *et al.*, 1999), lung adenocarcinoma (Kim *et al.*, 1999, 2000) and lowered levels of the receptor are observed in small cell lung carcinomas (de Jonge *et al.*, 1997). However, no studies have been conducted to determine the presence of any mutation in the TGF-β signalling pathway in MM.

Altered expression of TβRI or TβRII can also lead to TGF-β resistance in human tumours. A down-regulation of TβRI and II was found in a study on human colorectal carcinomas (Matsushita *et al.*, 1999). A decrease in TβRII expression was demonstrated in prostate cancer (Williams *et al.*, 1996). Complete loss of TβRI and TβRII were observed in a series of human T-cell lymphomas, along with a marked decrease in TβRII mRNA production (Kadin *et al.*, 1994).

Inactivating mutations or alterations in TGF-β signalling pathway intermediates leads to a TGF-β resistant state. Mutations in Smad4 are found in approximately 50% of pancreatic cancers (Chiao *et al.*, 1999) and also occur in gastric (Powell *et al.*, 1997) and colorectal carcinomas (Miyaki *et al.*, 1999). Yanagisawa *et al.* (2000) have described Smad2 and Smad4 mutations in human lung cancers. Hu *et al.* (2000) have demonstrated a decreased expression of Smad4 in ovarian cancer which may lead to a decrease in the intensity of TGF-β signalling. These mutations in the signalling pathway of TGF-β may suppress the anti-proliferative effects, whilst signalling through the MAPK and ERK pathways may promote pro-tumourigenic properties. For example, an inhibitor of TGF-β_1-induced MAPK and ERK signalling prevented human ovarian cancer cell invasion into matrigel, suggesting that TGF-β-induced signalling via these pathways promotes tumour invasion (Kobayashi *et al.*, 2003).

A study of TGF-β mediated inhibition of proliferation in human glioma cell lines demonstrated that Smad3 stimulated a cyclin dependent kinase inhibitor to stop the cell cycle, although this inhibitor was mutated in the majority of the carcinoma cell lines (Rich *et al.*, 1999). However, most of the cell lines retained other pro-tumourigenic responses to TGF-β, such as ECM production and the secretion of angiogenic cytokines, all of which would contribute to malignant growth. Kleeff *et al.* (1999) have shown that Smad7 mRNA levels are increased in human pancreatic cancer cells in comparison with normal pancreatic tissue. As Smad7 is an antagonist of TGFβ signalling, overexpression would prevent TGF-β signalling and inhibit anti-proliferative effects.

1.3.4 Inhibition of TGF-β as an anti-tumour strategy

As demonstrated above, there is a wealth of literature documenting the escape of tumour cells from the inhibitory effects of TGF-β on proliferation and apoptosis. In these cases the production of TGF-β by tumour cells and stromal cells contributes to late stage tumour progression and therefore inhibition of the elevated levels of TGF-β in MM or related downstream signalling elements could be beneficial in the treatment of this disease.

TGF-β activity can be inhibited by using recombinant soluble betaglycan (TβRIII) as a neutralising agent (Vilchis-Landeros *et al.*, 2001). Recombinant soluble betaglycan reduced tumour growth and metastasis in a model of human breast cancer (Bandyopadhyay *et al.*, 2002) with an associated decrease in angiogenesis. A soluble chimeric protein containing a portion of the TβRII when administered intraperitoneally increased mammary tumour apoptosis and reduced tumour cell intravasation and lung metastases in a transgenic murine model of breast carcinoma (Muraoka *et al.*, 2002). Soluble TβRII was also demonstrated to inhibit TGF-β signalling in human pancreatic cancer cell lines and reduced tumour growth and vascularisation in a subcutaneous murine model (Rowland-Goldsmith *et al.*, 2001).

Decorin is a small leucine-rich glycoprotein capable of binding to and neutralising TGF-β (Hildebrand *et al.*, 1994). Overexpression of decorin *in vivo* reduced TGF-β induced lung fibrosis (Kolb *et al.*, 2001). Decorin expressed ectopically in glioma

cells reduced TGF-β bioactivity and the expression of TGF-β_1 and TGF-β_2 mRNA and protein synthesis as well as strongly inhibiting tumour formation *in vivo* (Stander *et al.*, 1998).

Inducible antisense oligonucleotides to TGF-β_1 and TGF-β_2 have been shown to reduce the mRNA expression of these isoforms when transfected into murine MM cell lines (Fitzpatrick *et al,.* 1994). A murine MM cell line transfected with TGF-β_2 inducible antisense oligonucleotides exhibited reduced proliferation *in vitro* and an inhibition of tumour growth was observed when these cells were introduced into a murine model of MM (Marzo *et al.*, 1997). Also, in a Fisher 344 rat spontaneous MM cell line, antisense oligonucleotide-mediated inhibition of TGF-β_1 was shown to decrease the basal level of TGF-β_1 production and both the anchorage dependent and independent *in vitro* rate of proliferation (Kuwahara *et al.*, 2001). TGF-β is a potent inducer of collagen production, and collagen has been demonstrated to promote tumourigenesis (section 1.7). Therefore, TGF-β-induced collagen production may be important to MM growth. However, the effect of neutralising TGF-β on MM ECM production was not examined in any of the above studies.

Monoclonal antibodies have been hailed as "magic bullets" in the treatment of disease due to their ability to be delivered systemically and still target and neutralise specific molecules without harming healthy neighbouring cells (Gura, 2002). A large area of monoclonal antibody research is focussed on cancer treatment. The use of TGF-β monoclonal antibodies has primarily centred on reducing the pro-fibrotic effect of the TGF-β isoforms, and would thus be suited for examining the effect of TGF-β-induced collagen production in MM growth. For example, TGF-β_1 and -β_2 antibodies administered to a rat model of cutaneous scaring reduced collagen deposition (Shah *et al.*, 1995). The Cambridge Antibody Technology TGF-β_2 antibody (CAT-152) has been extensively characterised (Thompson *et al.*, 1999). This antibody inhibits TGF-β_2 function by associating with TGF-β_2, which prevents TβRII binding. CAT-152 reduced scarring in a rabbit model of glaucoma surgery and the same antibody reduced the ECM remodelling associated with TGF-β_2 in human cataract formation (Cordeiro *et al.*, 1999, Wormstone *et al.*, 2002). Also, antibodies to TGF-β_2, and all TGF-β isoforms, have also been successfully used to reduce the matrix expansion and

fibrosis in diabetic rat and mouse *in vivo* models (Hill *et al.*, 2001, Ziyadeh *et al.*, 2000).

In summary, TGF-β is a multifunctional cytokine with multiple tumour enhancing properties, and is found at elevated levels in MM. Inhibition of TGF-β in MM using inducible antisense reduced tumour growth through increased immune surveillance. The role of TGF-β-induced angiogenesis, apoptosis and ECM production in MM growth is unknown. Also, TGF-β potently stimulates collagen production, and collagen promotes tumour growth (see section 1.7). The use of TGF-β antibodies would provide insight into the importance of TGF-β-induced ECM production to MM growth. The principle components of the ECM, and their pro-tumourigenic properties are described in the following sections.

1.4 Extracellular matrix components

Animal cells secrete ECM components, which form a complex network of proteins and carbohydrates filling the space between cells 'gluing' tissue together. The ECM determines the mechanical properties of tissue, provides a scaffold for the attachment of cells and acts as a reservoir for many growth factors and cytokines controlling cell growth, migration and differentiation (Boudreau and Bissell, 1998). The ECM also provides a structure on which cells proliferate and migrate. The proteins of the ECM consist of several families of molecules, the bulk of which are collagens, interacting and binding with non-collagenous matrix, proteoglycans and glycosaminoglycans (Johansson, 1996).

1.4.1 Collagens

The collagens are a family of related proteins and are the major class of insoluble fibrous protein in the ECM and in the vertebrate body as a whole. All collagens contain a triple-helical domain composed of three polypeptide domains (α-chains) with a homologous amino acid sequence (Prockop *et al.* 1979). To date 41 distinct collagen α-chains have been identified, and these interact to form 27 known collagen isotypes (Pace *et al.*, 2003, Boot-Handford *et al.*, 2003).

The α-chains have a distinctive amino acid composition rich in L-proline and its derivative hydroxy-L-proline (collagen is 21% proline/hydroxyproline). These occur regularly in a repeating triplet of Gly-X-Y where approximately every third X is proline and every third Y is hydroxyproline (Miller, 1985). The pyrrolidine ring structure in proline enables each α-chain to coil into a left-handed helix consisting of 3 amino acids per turn. This geometry allows three α-chains to twist together forming a stable right-handed super-helical structure (Prockop *et al.*, 1979). Glycine, the smallest amino acid, and the peptide bonds of the collagen molecule, are in the centre of the triple helix making the helical region extremely resistant to proteolysis (Traub and Piez, 1971).

There are three main types of collagen. Firstly, fibril forming collagens such as collagen types I and III, confer tensile strength to tissue. Gram for gram collagen type I, the most abundant collagen, is stronger than steel (Darnell *et al.*, 1995). Fibrillar collagen monomers aggregate into ordered fibrils through covalent intermolecular bonds (Hulmes *et al.*, 1973) and demonstrate a characteristic 'D-spacing' pattern as observed under an electron microscope. This patterning is due to the staggering of neighbouring collagen molecules by approximately 300 nm (Prockop *et al.*, 1979).

Secondly are the non-fibril forming collagens, of which the most abundant is the basement membrane associated collagen type IV (Madri and Furthmayr, 1980). These collagens provide the major structural scaffold of all basement membranes and form an open meshed two-dimensional network. The triple helix sequence of collagen type IV is interrupted by non-triple helical sequences, which increase the flexibility of the molecule (Timpl, 1989).

Lastly are the fibril-associated collagens with interrupted triple helices (FACIT) such as collagen types IX, XII & XIV. These collagens do not form fibrils themselves but are instead associated with various fibrillar collagens and are thought to act as molecular bridges between different ECM components (Sugre *et al.*, 1989, Shaw and Olsen, 1991).

Collagen has been shown to influence cell migration, proliferation and angiogenesis, all of which are processes that would stimulate tumour growth. For example, in a skin wound the provisional wound matrix is rich in collagens type I, III and IV. For re-epithelialization of the wound to occur the migration of keratinocytes is required. Human keratinocytes have been shown to have a dramatic rate of migration to collagen types I and IV (Woodley *et al.*, 1988). Additionally, collagen type IV can sequester pro-angiogenic factors such as VEGF and bFGF, which can be subsequently released by MMP-2 and –9 to initiate tumour angiogenesis (Bergers *et al.*, 2000). Also, fragments of collagen type I are chemotactic for endothelial cells (Palmieri *et al.*, 2000). Collagen has also been demonstrated to have many pro-tumourigenic properties, which are reviewed in section 1.7.

1.4.2 Collagen biosynthesis

Figure 1.4 summarises the key steps in collagen biosynthesis. Procollagen DNA is transcribed to pre-mRNA in the cell nucleus and modified by the capping of the 5' end with a methylated G and the polyadenylation of the 3' end (Bateman *et al.*, 1996). The intronic sequences are spliced out to form a functional mature mRNA that is translated to protein on the membrane-bound polysomes in the rough endoplasmic reticulum (RER). The preprocollagen α-chain contains an N-terminal hydrophobic signal peptide which is believed to facilitate movement across the RER. During translation, or shortly after polypeptide synthesis, this peptide is proteolytically cleaved (Chambers and Laurent, 1997). The resultant procollagen α-chain is extensively processed through a series of co- and post-translational modifications, including the hydroxylation of proline and lysine residues in the Y position of the Gly-X-Y triplet. Hydroxylation catalysed by prolyl 4-hydroxylase and lysyl hydroxylase result in 4-hydroxyproline and hydroxylysine respectively, and require the essential co-factors ferrous iron and ascorbic acid (Kivirikko *et al.*, 1989). Collagen α-chains must each contain at least 90 residues of 4-hydroxyproline to form a stable triple helix at 37°C. Without ascorbate there is insufficient hydroxylation and the α-chains are unable to form normal fibrils at body temperature (Rosenbloom *et al.*, 1973; Berg and Prockop, 1973). The physiological importance of proline and lysine hydroxylation is evident in scurvy, where ascorbate deficiency leads to unstable

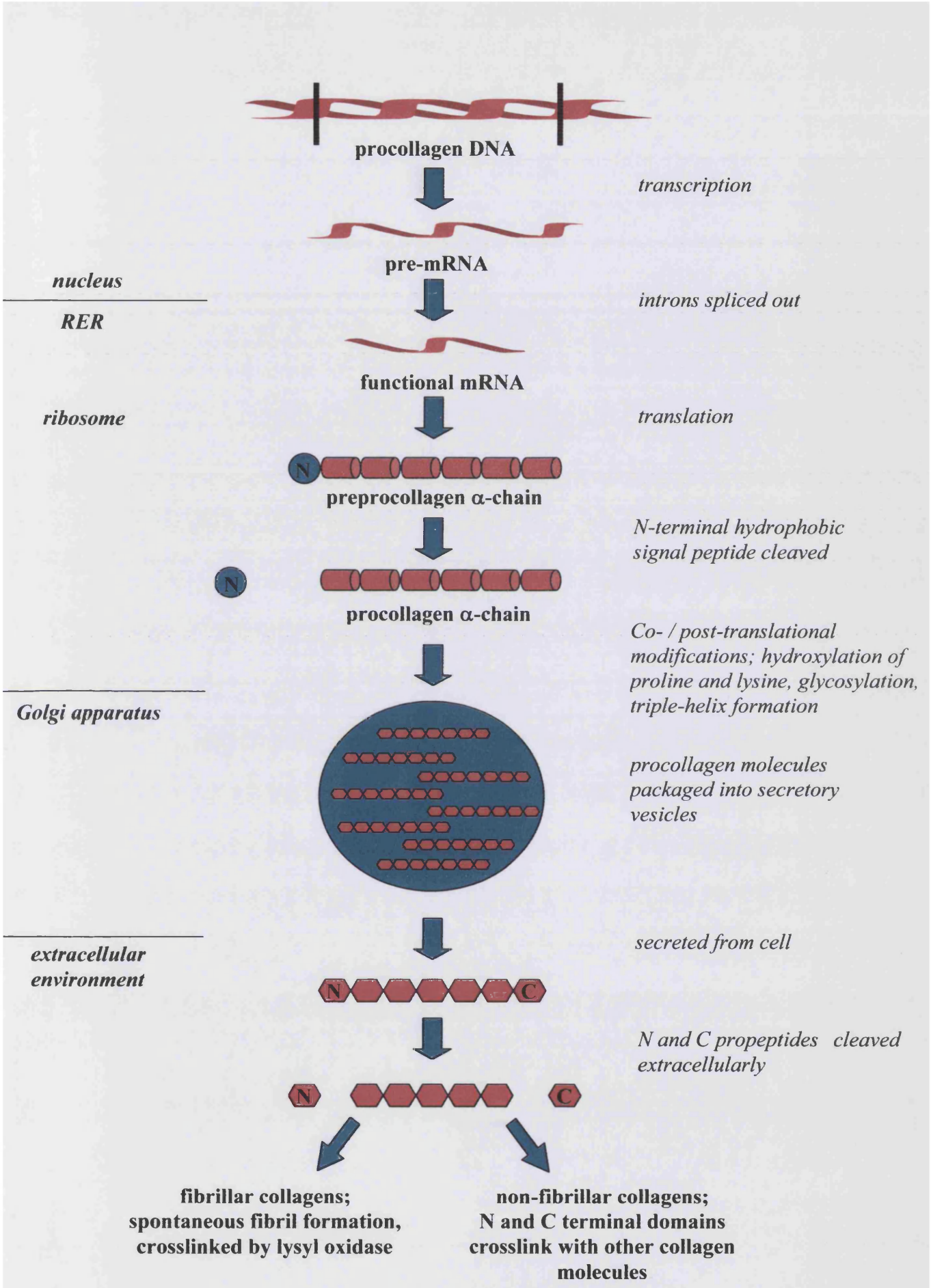


Figure 1.4 Flow chart indicating key steps in collagen biosynthesis

collagen triple helices resulting in weakened bone and dentin, impaired wound healing and blood capillary breakdown with associated haemorrhaging.

The hydroxylation of proline residues in the X position occurs by prolyl 3-hydroxylase, but this is dependent on the neighbouring Y position being occupied by 4-hydroxyproline (Tryggvason *et al.*, 1976, Risteli *et al.*, 1977). Glycosylation ofcertain hydroxylysine residues also occurs. The specific function is unknown but the attached carbohydrate is thought to adjust the biological properties of the collagen (Kivirikko and Myllyla, 1979, Bateman *et al.*, 1996). Finally, mannose-rich oligosaccharides are transferred onto the c-propeptides of fibrillar collagens and some domains in non-fibrillar collagens (Kaplan *et al.*, 1987, Hirschberg and Snider, 1987).

Once the post-translational modifications are completed within the ER, procollagen assembly occurs. The c-propeptide domains of three α-chains fold individually and trimerise through association of the c-propeptide domains in a zipper-like fashion to form a fully folded procollagen molecule (Prockop, 1990). Newly synthesised procollagens in the ER are associated with molecular chaperones, suggesting their importance in the procollagen folding pathway (Lamande and Bateman, 1999). Binding protein (BiP), GRP94 and protein disulphide isomerase (PDI; also a subunit of prolyl 4-hydroxylase) catalyse the folding of the c-propeptide domain through intrachain disulphide bond formation, and HSP47 assists the formation of the triple helix (Lamande and Bateman, 1999).

Procollagen molecules are transported from the RER to the Golgi apparatus where they are packaged into secretory vesicles. During secretion the N- and C-propeptides are enzymatically cleaved by procollagen N- and C-proteinase respectively (Tuderman and Prockop, 1982, Tanzawa *et al.*, 1985). Removal of the C-propeptide results in an approximate 10,000-fold decrease in the solubility of procollagen and is essential for fibril formation (Parsons *et al.*, 1999).

Fibrillar collagens spontaneously assemble into fibrils (Wood, 1960), initially held together by weak electrostatic interactions. Cross-link formation is crucial for the physical and mechanical properties of the fibrils and provides collagen fibrils with

their characteristic high tensile strength (Bateman *et al.*, 1996). Lysyl oxidase facilitates cross-linking by catalysing the formation of aldehydes between lysine or hydroxylysine residues on neighbouring fibrils (Eyre *et al.*, 1984).

Non-fibrillar collagens contain similar residues to the fibrillar collagens so lysyl oxidase catalysed covalent cross-linking may also occur (Henkel and Glanville, 1982). Collagen type IV is covalently cross-linked, resulting in the formation of an open meshed network structure as observed in basement membranes (Yurchenco and Furthmayr, 1984).

1.4.3 Non-collagenous matrix proteins

The ECM is also composed of non-collagenous components, several of which have also been demonstrated to be pro-tumourigenic (section 1.7). Fibronectins are a family of related proteins derived from alternatively spliced pre-mRNA from a single gene (Tamkun *et al.*, 1984, Kornblihtt *et al.*, 1985). Fibronectins have a demonstrated importance in cell migration and differentiation, wound healing, blood coagulation and tumour formation and metastasis (Hynes, 1990).

Tenascins are large and complex extracellular matrix proteins consisting of three members, C, R and X (Bristow *et al.*, 1993). Tenascins have a limited distribution in the body, with each form expressed at different locations. Tenascin C is an important ECM protein in embryogenesis, wound healing and in tumours, tenascin R is found exclusively in the central nervous system and tenascin X has been observed in muscle and around blood vessels (Johansson, 1996).

Vitronectin, along with fibronectin, is the major cell adhesion protein in blood plasma and serum (Johansson, 1996). Vitronectin has been associated with the surface of neural crest cells (Delannet *et al.*, 1994) and in the matrix of vessel walls (Guettier *et al.*, 1989).

Elastin and fibrillins provide elasticity to tissues that are subject to repetitive distension, although their distribution is not confined to these tissues alone (Cleary and Gibson, 1997). Elastin fibres are composed of a disorganised core of elastin,

surrounded by a lattice of microfibrils (Rosenbloom *et al.*, 1993). Fibrillins are virtually identical in structure to elastins (Arteaga-Solis *et al.*, 2000).

The proteoglycans (PGs) are a family of highly glycosylated multi-domain core proteins. PGs have distinct protein backbones with covalently attached carbohydrate rich glycosaminoglycans (GAGs). Due to the negative charge and varying pore sizes of PGs and the attached GAG chains these molecules are able to act as molecular sieves controlling the movement and storage of signalling molecules according to size and charge (Ruoslahti and Yamaguchi, 1991).

Decorin and biglycan belong to a group of small leucine rich PGs and are capable of binding and holding TGF-β in a neutralised state (Hildebrand *et al.*, 1994, Yamaguchi *et al.*, 1990, Kolb *et al.*, 2001). They are commonly found in most connective tissues and share a similar structure; decorin with one binding site for GAG attachment and biglycan containing two (Fosang and Hardingham, 1996). Decorin and biglycan have been implicated in connective tissue reorganisation and organ differentiation during mouse embryogenesis (Wilda *et al.*, 2000). Decorin has also been shown to bind fibrillar collagens (Scott and Orford, 1981).

The GAG hyaluronan is abundant in the early stages of ECM production by most mammalian cells and is found in early embryogenesis (Fraser and Laurent, 1997). Mesothelial cells in culture synthesis hyaluronan, which is upregulated following injury of the mesothelial layer (Yung *et al.*, 2000). The elastic properties of hydrated hyaluronan allow the maintenance of shape in soft connective tissues that have applied stresses. Hyaluronan functions as a space-filler in tissues and also has a protein and cell binding role (Delpech and Delpech, 1984, Aruffo *et al.*, 1990). Additionally, hyaluronan may also protect cells from viral infections, the cytotoxic effects of lymphocytes and may be important in cell differentiation (Heldin and Pertoft, 1993). Hyaluronan may also prevent tumour cell dissemination and growth of ovarian tumour cells in the peritoneal cavity (Jones *et al.*, 1995). Furthermore, MM pleural effusions containing elevated levels of hyaluronan correlated with longer patient survival (Thylen *et al.*, 2001), suggesting that hyaluronan has a suppressive role in MM growth.

1.5 Integrins

Tumour growth is dependent upon the proliferation and migration of cells through the surrounding stromal tissue. Cell movement occurs by the application of a force against a surface, and adhesion to a solid surface is the first step in cell migration. The integrins are a major family of heterodimeric transmembrane receptors consisting of α and β subunits. Integrins connect the cell to the external ECM, anchor the cytoskeleton to the plasma membrane on the inside of the cell and serve as receptors for signal transduction events leading to cell proliferation, survival / apoptosis, shape, motility and gene expression (Hynes, 2002).

A recent review of the literature reported 18 α and 8 β mammalian subunits that are known to assemble into 24 distinct integrins, each having a specific function and varying ligands (Hynes, 2002). Activation of integrins must occur for binding and adhesion to the ECM. Integrins have various states of affinity for their ligands with bi-directional signal transduction. The level of affinity can be regulated by extracellular factors, 'outside-in' signalling, or by intracellular processes, 'inside-out' signalling (Schwartz and Ingber, 1994, Holly *et al.*, 2000). These signalling pathways provide a sophisticated attachment-detachment mechanism controlling the extent of cell adhesion to the ECM.

Normal mesothelial cells produce $\alpha_{1\text{-}6}$, β_1 and β_3 integrin subunits (Boylan *et al.*, 1995, Leavesley *et al.*, 1999, Liaw *et al.*, 2001). However, malignant cells express a different integrin profile, MM produces $\alpha_{1\text{-}6}$, α_v, β_1, β_3 and β_4 (Boylan *et al.*, 1995, Barth *et al.*, 1997, Klominek *et al.*, 1997, Koukoulis *et al.*, 1997, Giuffrida *et al.*, 1999). Also, variation in integrin expression is observed between the different MM cell phenotypes. Epithelial-like MM cell lines express more intense staining for the α_6, α_v and β_1 integrin subunits than fibroblast-like MM cell lines (Koukoulis *et al.*, 1997, Giuffrida *et al.*, 1999). The variation in integrin expression may be responsible for the differences observed in disease progression between MM morphologies. (Parker and Neville, 2003)

Palecek *et al.* (1997) established that the rate of cell migration is dependent upon the level of cell adhesion to the ECM, controlled by the variables of ECM density, integrin expression and activation. The rate of migration against the extent of

adhesion could be plotted as an approximate bell-shaped curve (figure 1.5). A low level of adhesion results in a slow rate of migration as there is not enough traction for the cells to move significantly (figure 1.5 A). Increasing the adhesion up to an optimal level results in the highest rate of cell migration (figure 1.5 B). Further cell attachment leads to impaired mobility (figure 1.5 C), probably due to the inability to detach. Depending on the starting position on this curve, altering integrin expression / activation or changing the ECM density may have different effects on migration. Migration would be enhanced by increasing the level of adhesion when less than optimal, but when optimal, further adhesion would decrease the rate of migration.

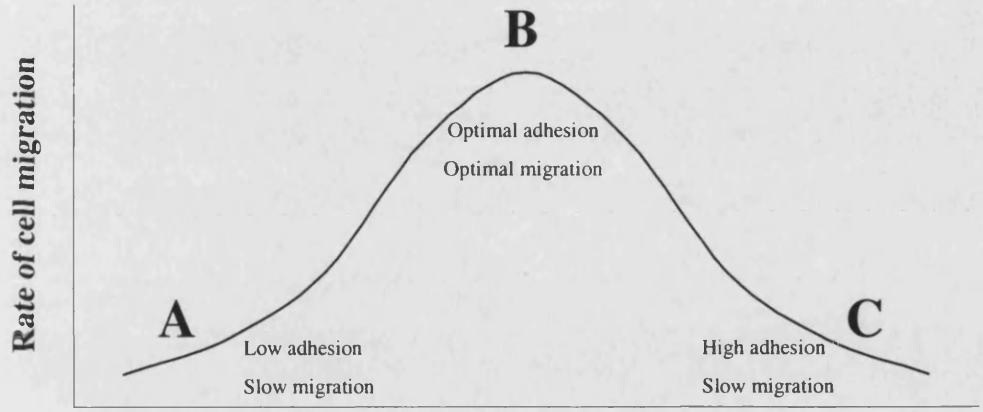


Figure 1.5 The rate of cell migration is dependent upon the level of cell adhesion to the surrounding ECM (adapted from Holly *et al.*, 2000).

In the literature there are no obvious trends in the quantitative and qualitative changes of integrin expression / activation amongst different tumour types. For example, bladder carcinomas exhibit increased expression of α_1 integrin (Liebert *et al.*, 1994), whereas decreased levels of α_1 are found in human melanoma and breast carcinomas (Schadendorf *et al.*, 1993, Gui *et al.*, 1994). The above model of the level of adhesion affecting cell migration may explain how different integrin profiles could lead to a similar malignant phenotype.

The $\alpha_6\beta_4$ integrin (laminin binding) is heavily implicated in epithelial tumours (Mercurio and Rabinovitz, 2001). Increased expression of $\alpha_6\beta_4$ is observed in skin

carcinoma (Tennenbaum *et al.*, 1993), bladder cancer (Grossman *et al.*, 2000) and new expression is seen in thyroid tumours (Serini *et al.*, 1996). Overexpression of $\alpha_6\beta_4$ increases the formation of papilloma and squamous cell carinoma (Watt, 2002).

The β_1 subunit is predominately associated with collagen and laminin adherence. β_1 integrin expression is higher and it's surface distribution altered in human breast cancer cells compared to non-malignant cells (Weaver *et al.*, 1997). When attached to ECM components (fibronectin, laminin, collagen type IV) the β_1 integrin stimulates survival signals preventing the chemotherapy-induced apoptosis of small cell lung cancer cells (Sethi *et al.*, 1999). Human hepatocellular carcinoma has an increased expression of β_1 integrin compared to non-cancerous liver tissue (Ozaki *et al.*, 1998), the β_1 integrin protecting tumour cells from chemotherapy-induced apoptosis by a MAPK dependent pathway (Zhang *et al.*, 2002).

The altered expression of integrins in the different tumour types above is likely to contribute to the malignant properties of cell motility, proliferation and metastasis. This evidence suggests that the dysregulation of cell-ECM interactions are important in malignant transformation.

1.6 Matrix metalloproteinases

As well as penetrating and invading the surrounding ECM in order to grow, malignant tumours must also have a mechanism to detach from the ECM to metastasise to distant sites. There are several families of proteins, including serine proteases, cathepsins and matrix metalloproteinases (MMPs), which are capable of cleaving various components of the ECM allowing cell invasion and detachment. MMPs are a group of over 20 enzymes capable of degrading the ECM, and are the only enzymes that can denature and digest fibrillar collagens (Rintoul and Sethi, 2001). MM has been demonstrated to produce MMP-1, MMP-2, MMP-3, MMP-9 as well as the natural antagonists of MMPs, TIMPs 1, 2 and 3 by RT-PCR. Several of the MM cell lines also expressed MMP-7 and MMP-10 (Liu *et al.*, 2001).

It is widely believed that elevated levels of MMPs are associated with more invasive and metastatic tumours. Human fibrosarcoma cells stably transfected with MMP-13

have an enhanced invasive capacity through collagen type I gels and matrigel (Ala-Aho *et al.*, 2002), an effect blocked by addition of a synthetic MMP inhibitor or TIMP-3. Expression of MMPs has been demonstrated in human lung carcinomas by *in situ* hybridisation (Zucker *et al.*, 1992, Kossakowska *et al.*, 1996), Northern blotting (Sato *et al.*, 1992) and ELISA (Iizasa *et al.*, 1999). In an animal model of experimental melanoma, MMP inhibitors were shown to reduce both tumour growth and secondary lung metastases in a dose-dependent manner (Naglich *et al.*, 2001). The use of MMP inhibitors in the treatment of renal and lung carcinoma patients is currently being investigated in ongoing clinical trials (Falardeau *et al.*, 2001, Wojtowicz-Praga *et al.*, 1998).

The view that MMPs only role is to degrade ECM has been altered in recent years with the discovery that MMPs are dynamic molecules with many cellular functions (McCawley and Matrisian, 2001). The ECM acts as reservoir, sequestering growth factors and cytokines (Boudreau and Bissell, 1998), and the proteolysis of ECM by MMPs can release fragments with biological activities (Werb, 1997). MMPs–2, -3 or -7 can cleave decorin and release TGF-β_1 (Imai *et al.*, 1997). MMPs have also been demonstrated to cleave and activate growth factors. Yu and Stamenkovic (2000) have shown that MMPs-2 and -9 are capable of activating pro-TGF-β_1 to localise TGF-β_1 to the cell surface. Vascular endothelial growth factor (VEGF) release mediated by MMP-9 has been shown to be vital for angiogenesis in a mouse model of pancreatic islet tumours (Bergers *et al.*, 2000). MMP-3 and -7 have also been shown to cleave E-cadherin generating a fragment that can stimulate Madin-Derby canine kidney cells to migrate and invade collagen type I gels (Noe *et al.*, 2000).

The literature cited above suggests that many of the MMPs produced by MM may be important in cell migration, invasion into the surrounding ECM and in the release of cytokines that stimulate angiogenesis and ECM synthesis, all of which may promote tumour growth.

1.7 The role of the extracellular matrix in tumourigenesis

To grow beyond a minimal size of 1 to 2 mm, all solid tumours require ECM (Folkman, 1985), with interactions between tumour cells and the ECM crucial to

cancer progression. The ECM may account for over 90% of total tumour mass in fibrous tumours, although other cancers such as medullary carcinomas have minimal stroma (Dvorak, 1986). Inflammatory cell infiltration rarely accounts for more than a few percent of tumour mass.

Although tumour specific differences exist, common features are apparent between all invasive and metastatic tumours, including adhesion to the ECM through cell ECM-interaction, invasion and migration through the tumour stroma (Varani, 1987). The ECM has a demonstrated role for invasion. For example, carcinoma cells implanted into granulation tissue have a more invasive phenotype (Dingemans *et al.*, 1993, Gabbert, 1985). ECM proteins can also act as a reservoir to release growth factors increasing tumourigenesis (Wernert, 1997). The role of several common ECM proteins in the tumour growth of different malignancies is summarised in table 1.1. It has been proposed that prevention of the generation of tumour stroma may be useful in cancer treatment, depriving tumours of support, survival and growth factors (Dvorak, 1986). MM has a dense ECM, and therefore this approach may be applicable to MM treatment.

1.7.1 ECM components associated with malignant mesothelioma

Although a characteristically fibrous tumour rich in ECM, the role of ECM proteins in MM have not been fully elucidated. MM cell lines migrate to fibronectin, laminin and collagen type IV *in vitro*, an effect that can be inhibited by incubation with blocking integrin β_1 antibodies (Klominek *et al.*, 1997). Increased levels of hyaluronan are observed in MM which contributes to invasive growth in soft agar (Li and Heldin, 2001). Also, fibrinogen deposition is a feature associated with MM (Wojtukiewicz *et al.*, 1989). Furthermore, EGF, IGF and PDGF stimulate MM cells to produce proteoglycans (Sykrokou *et al.*, 1999), the expression of which influences cell morphology and aggregation (Dobra *et al.*, 2000). Interestingly, mesothelioma cell cultures also have a greater capacity to produce collagen than benign mesothelial cells (Castor *et al.*, 1969, Behbehani *et al.*, 1982, Whitaker *et al.*, 1984, Craighead *et al.*, 1987), suggesting a role for collagen in MM. However, the function of collagen in MM is unknown and further studies are required to determine the effect of inhibiting collagen production on MM cell proliferation and tumour growth.

Three different MM cell morphologies have been demonstrated *in vitro* and *in vivo*; epithelial-like with a cobblestone appearance, fibrosarcomatous (fibroblast-like, elongated spindle shaped) and biphasic (mixed epithelial and fibroblast-like morphology). Fibroblast-like cell morphology is associated with a worse prognosis (Fusco *et al.*, 1993). Fibroblast like MM cell lines produce more matrix PGs such as decorin and biglycan than epithelial-like MM cell lines (Dobra *et al.*, 2000), suggesting a role for these PGs in more aggressive tumour growth.

ECM Component	***Evidence for pro-tumourigenic properties***
Collagen	Association of collagen type I and III with breast tumours (Kauppila *et al.*, 1998) Collagen type I and III expression increased in tobacco smoke-induced hamster lung cancers (Laitakari and Stenback, 2001) Human colorectal malignancies contain increased collagen type I and V (Fischer *et al.*, 2001) Collagen type IV protected small cell lung cancer cells (SCLC) from chemotherapy-induced apoptosis (Sethi *et al.*, 1999) Collagen type IV increased astrocytoma cell migration (Berens *et al.*, 1994) Desmoplasia around breast carcinoma contained increased collagen V (Barsky *et al*, 1982) Desmoplasia associated with increased occurrence and metastases of skin cancers (Breuninger *et al.*, 1997) Increased collagen & prolyl hydroxylase in breast tumours (Al-Adnani *et al.*, 1975) Inhibition of collagen production with a proline analogue reduced breast carcinoma growth *in vitro* and *in vivo* (Lewko *et al.*, 1981)

Table 1.1 ECM components associated with tumourigenesis

Fibronectin	Enhanced breast cancer cell proliferation (Simpson-Haidaris & Rybarczyk, 2001) Increased the mitogenic and migratory effects of VEGF, PDGF and bFGF on breast carcinoma cells (Elliot *et al.*, 1992, Miralem *et al.*, 2001) Increased glioma cell migration (Enam *et al.*, 1998) Required for mammary tumours to form colonies (Sauliner *et al.*, 1996, 1997)
Hyaluronan	Increased invasion of human glioma cells (Radotra and McCormick, 1997) Increased migration of melanoma cells (Yoshinari *et al.*, 1999) Increased levels associated with breast carcinoma (Madan *et al.*, 1999)
Laminin	Increased proliferation and migration of corticotroph tumour cells (Kuchenbauer *et al.*, 2001) Increased migration of astrocytoma and glioma cells (Berens *et al.*, 1994, Mahesparan *et al.*, 1997) Prevented chemotherapy-induced apoptosis of SCLC (Sethi *et al.*, 1999)
Osteopontin *	Upregulated in murine skin carcinoma and enhanced cell adhesion, migration, invasion and *in vivo* growth (Craig *et al.*, 1990, Philip *et al.*, 2001)
Mixture of basement membrane proteins	Increased colon cancer cell proliferation and migration (Ohtaka *et al.*, 1996) Increased growth of malignant breast epithelial cells (Petersen *et al.*, 1992) Increased breast carcinoma incidence and growth *in vivo* (Noel *et al.*, 1994) Increased human lung cancer cell proliferation and malignant phenotype (Pavelic *et al.*, 1992) Increased human hepatocarcinoma and ovarian cancer cell migration and proliferation (Vlodavsky *et al.*, 1980, Crickard *et al.*, 1983)

Table 1.1 continued. ECM components associated with tumourigenesis

* Osteopontin is a glycosylated phosphoprotein which has been shown to bind collagen type I and fibronectin

The experimental evidence described in this section demonstrates that the ECM has growth promoting effects on tumours, increasing migration, proliferation, invasive capacity, aggregation and colony formation *in vitro* and the incidence and ability to metastasise *in vivo*. This suggests that modulation of ECM production, of which collagen is a major component, may be useful in the control of MM tumour growth. The next section describes several strategies for inhibiting collagen production as a means of attenuating MM tumour growth.

1.8 Inhibition of collagen biosynthesis

Overexpression of MMPs, the natural enzymes capable of degrading collagen, would reduce the amount of collagen present in the ECM. However, MMPs are involved in the activation of growth factors and can stimulate the invasion and migration of cells (section 1.6). In addition, MMPs act at the protein level, degrading ECM after it has been produced and therefore giving an opportunity for cell-matrix interactions to occur. The use of an antagonist with minimal non-specific effects would be more desirable.

As collagen synthesis is a complex multi-step process (see figure 1.4), its production could be inhibited at several points. These include procollagen gene transcription, translation of mRNA to protein, hydroxylation or glycosylation of procollagen α-chains, intracellular transport, cleavage of the procollagen terminal peptides or chain polymerisation and fibril cross-linking.

Procollagen gene transcription can be inhibited by glucocorticoids (Cutroneo *et al.*, 1981, Dik *et al.*, 2003), and glucocorticoids have been shown to reduce the collagen accumulation associated with instillation of bleomycin into rat lungs (Sterling *et al.*, 1982). Antisense oligonucleotides which hybridise to double stranded DNA can also inhibit gene transcription, and those that target mRNA prevent translation (see review by Helene and Toulme, 1990).

Proline hydroxylation can be prevented by bivalent cations, such as Zn^{2+}, which are believed to bind essential co-factors (Anttinen *et al.*, 1984). Chemical inhibitors of prolyl hydroxylase have been developed (Hales and Beattie, 1993, Baader *et al* 1994,

Matsumura *et al.*, 1997), although it has been noted that the high affinity of prolyl hydroxylase for its substrate is an obstacle in reducing the activity of the enzyme (Fuller, 1981).

β-aminopropionitrile (BAPN) binds irreversibly to and inactivates lysyl oxidase (Tang *et al.*, 1983). Several studies have appeared in the literature demonstrating the ability of BAPN to reduce collagen deposition in models of fibrotic disease (Zuckerman *et al.*, 1980, Kagan, 1994, Ledwozyw, 1995).

Proline analogues have been used to disrupt the translation of procollagen mRNA to protein. Uitto and Prockop (1975) demonstrated that proline analogues were incorporated into nascent procollagen α-chains preventing the normal triple-helical conformation leading to an increased rate of degradation. The analogues used decreased the rate of total protein synthesis but the effects were small compared to the marked reduction in extracellular procollagen production.

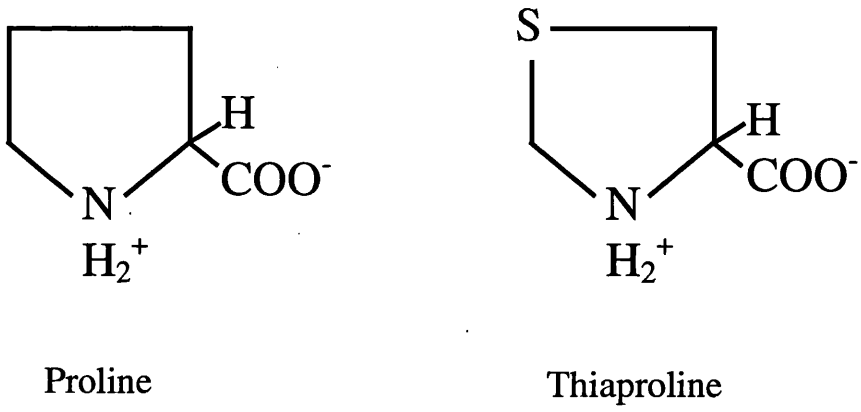


Figure 1.6 The dipolar ion form (neutral pH) of proline and thiaproline. The γ carbon (C-4) of proline is substituted to a S in thiaproline, preventing chain elongation of the growing polypeptide chain.

The proline analogue thiaproline differs from proline by the substitution of the γ carbon (C-4) to a sulphur atom (see figure 1.6 above). Once incorporated into a growing polypeptide chain, thiaproline prevents further elongation leading to truncated peptides which are degraded (Uitto and Prockop, 1974, Busiello *et al.*, 1979). Additionally, as the γ carbon on proline is hydroxylated by prolyl

hydroxylase, incorporation of thiaproline into procollagen α-chains would decrease the amount of hydroxyproline and may therefore destabilise the triple helical structure. Thiaproline has been used as an inhibitor of collagen production and has been shown to inhibit collagen accumulation in both the glomerular basement membrane and heart ventricles of diabetic db/db mice (Lubec *et al.*, 1994 and 1997) with no apparent toxicity at the doses used. In these studies thiaproline was given orally to the mice as part of their diet. The combination of the ease of *in vivo* administration, specificity and demonstrated efficiency in reducing collagen accumulation make thiaproline suitable for investigating the role of collagen in MM tumour growth.

1.9 Summary and aims of study

The literature reviewed in this introduction has highlighted several key points. MM is an extremely aggressive tumour, with an unknown pathogenesis, inadequate therapy and very poor patient prognosis. A better understanding of MM biology may lead to improved treatment. Evidence in the literature supports a strong role for the ECM in malignant cell proliferation, migration, invasion and *in vivo* tumour growth. Although a characteristically fibrous tumour shown to express collagen, hyaluronan, and fibronectin amongst others, the role of the ECM in MM proliferation and tumourigenesis is unknown. Previous studies in other tumours suggest that collagen, the predominant ECM component, will promote MM growth, and therefore strategies aimed at inhibiting collagen production would be of use. In other carcinomas, inhibition of collagen production or the blocking of cell-collagen interactions has been shown to decrease tumour growth. The proline analogue thiaproline is specific, non-toxic and decreases the accumulation of collagen *in vivo*.

Furthermore, in many cancers TGF-β is produced at elevated levels and there is aberrant expression of TGF-β receptors or there is dysregulation in TGF-β intracellular pathways leading to persistent signalling. These alterations have been demonstrated to contribute to the tumourigenicity of malignant cells. TGF-β has many pro-tumourigenic effects, such as enhancing cell proliferation, migration, invasion, immune suppression, angiogenesis and ECM production. Additionally,

TGF-β is a highly potent inducer of collagen production, and inhibition of TGF-β with neutralising antibodies has been demonstrated to attenuate fibrosis *in vivo*. MM also produces elevated levels of TGF-β compared to normal mesothelial cells and other primary lung carcinomas. Therefore, it was hypothesised that TGF-β-induced collagen production may be stimulating MM tumour growth, as both collagen and TGF-β have pro-tumourigenic properties. The use of TGF-β antibodies would be appropriate for studying the effect of TGF-β-induced collagen production on MM tumour growth.

The above observations led to the formulation of the global thesis hypothesis:

The ECM is vital to the progression of malignant mesothelioma with TGF-β-induced collagen production stimulating cell proliferation and tumour growth

Although previous studies have assessed the role of the ECM and TGF-β separately in various tumour settings, there have been no reports investigating the importance of collagen and it's production by TGF-β in the growth of MM. The specific aims of this thesis are to:

(i) Measure the levels of collagen and TGF-β produced by normal and malignant mesothelial cells lines *in vitro*.

This aim will be achieved by measuring hydroxyproline as an assessment of collagen production in MM cell cultures using High Performance Liquid Chromatography (HPLC). The amount of TGF-β produced by MM cell cultures into conditioned media will be measured using the mink lung epithelial cell TGF-β bioassay, consisting of the TGF-β response element PAI-1 fused to a luciferase reporter gene.

(ii) Determine the importance of collagen production for MM cell proliferation *in vitro,* and for tumour growth in an established subcutaneous animal model by using thiaproline to inhibit collagen production.

Cells will be cultured on differing collagen substrates and proliferation assessed to determine any effect on cell proliferation. The proline analogue thiaproline will be used to inhibit collagen production *in vitro* and the effect on cell proliferation observed. Thiaproline administered *in vivo* will be used to assess the effect of inhibiting collagen production on tumour growth. Tumour tissue will be analysed by HPLC to determine the collagen content.

(iii) Evaluate the effect of TGF-β neutralising antibodies on MM cell proliferation, collagen production and tumour growth *in vivo.*

A panel of TGF-β neutralising antibodies will be assessed for their specificity and potency to neutralise TGF-β_1, -β_2 and -β_3. The effect of specifically inhibiting TGF-β isoforms with these antibodies on MM cell proliferation, collagen production and tumour growth *in vivo* will be assessed. Tumours will be analysed for collagen content by HPLC to determine whether TGF-β-induced collagen production plays a role in MM tumour growth.

Chapter Two

Materials and Methods

2.1 Maintenance of cell lines in tissue culture

Human Malignant Mesothelioma (MM) cell lines (JU77, LO68, NO36 and ONE58; characterised by Manning *et al.*, 1991) and murine MM cell lines (AB1, AB22 and AC29; derived by Davis *et al.*, 1992) were provided as a generous gift by Prof. B.W.S. Robinson (University Department of Medicine, Queen Elizabeth II Medical Centre, Perth, Western Australia). The normal human mesothelial cells (NM20) used were obtained and subsequently characterised from effusions of patients with heart failure (Heldin *et al.*, 1992, Langerak *et al.*, 1996).

Cells were maintained in 75 cm^2 tissue culture flasks (Helena Biosciences, Tyne & Wear, UK) in 10 ml of standard growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS; Imperial Laboratories, Andover, UK), L-glutamine (4 mM; Invitrogen), penicillin (200 units/ml), and streptomycin (200 units/ml). Flasks were incubated at 37°C in a humidified atmosphere of air containing 10% CO_2.

The malignant cell lines used exhibited loss of contact inhibition and when dense would pile up and continue growing over the cell monolayer. Cultures were judged to be confluent when cells were touching and fully surrounded by neighbouring cells but still existing as a monolayer. Upon visual confluence cells were passaged into new culture flasks. The growth medium was aspirated and the cell layer washed twice with 10 ml of phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) to remove any residual medium. Cells were incubated with a 1 ml solution of trypsin (0.05%, w/v) / ethylenediaminetetraacetic acid (EDTA, 0.02%, w/v; Invitrogen) at 37°C until they detached from the flask surface. The cells were observed under an Olympus TCK-2 inverted phase contrast light microscope (Olympus Optical Co., Tokyo, Japan) to ensure adequate detachment. The trypsin was neutralised with 9 ml of standard medium and the cell suspension split at an appropriate ratio depending on the cell line and transferred to new flasks containing 10 ml of standard medium.

Cell number was determined using an improved Neubauer haemocytometer (BDH-Merck, Lutterworth, UK). For experimental procedures, the required number of cells

were transferred to a sterile 50 ml polypropylene centrifuge tube and centrifuged (300 x g, 5 min, 4°C) using a bench centrifuge (MSE Mistral 3000, Loughborough, UK), the supernatant discarded and the cell pellet resuspended at the appropriate concentration in DMEM containing 1% (v/v) FBS, prior to plating. All experiments were conducted in 1% serum. Serum free conditions resulted in loss of cell adhesion to tissue culture plastic and cell death.

2.1.1 Freeze storing of cells

Cells were detached from flasks and pelleted as described previously. The cell pellet was resuspended in DMEM containing 20% (v/v) FBS. An equal volume of solution containing 20% (v/v) dimethyl sulphoxide (DMSO; Sigma Aldrich, Poole, UK) / 20% (v/v) FBS in DMEM was added dropwise to the cell suspension. The cells were transferred to cryovials (Nalge Nunc, Naperville, USA) and allowed to slowly freeze overnight at -70°C before being transferred into liquid nitrogen.

2.1.2 Thawing of cells

Cells were recovered from liquid nitrogen storage by heating in a 37°C waterbath. The cells were then transferred to a flask containing standard growth medium and incubated at 37°C. The growth medium was aspirated and replaced the following day to remove the DMSO solution.

2.1.3 Isolation of murine normal mesothelial cells

Normal mesothelial cells were isolated from the anterior peritoneal wall of 8 week-old female CBA mice (Harlan, Oxon, UK) for comparison with syngeneic malignant cells. Animals were sacrificed by cervical dislocation. The skin on the abdomen was separated from the underlying abdominal wall and the abdominal wall excised and placed into a 50 ml centrifuge tube containing 25 ml of trypsin (0.25%, w/v) / EDTA (0.02%, w/v). Tissue from three mice was placed in each tube of trypsin / EDTA. The tubes were shaken for 30 min at 37°C in a shaking incubator, the intact tissue removed and discarded and the remaining cell suspension centrifuged (300 x g, 5 min, 4°C) in a bench centrifuge. The supernatant was discarded and the pellet resuspended in 10 ml of mesothelial cell growth medium consisting of DMEM supplemented with 15% (v/v) FBS, 4 mM L-glutamine, 5 ng/ml epidermal growth factor (Roche

Diagnostics, Lewes, UK), 0.4 μg/ml hydrocortisone (Sigma Aldrich), 200 units/ml penicillin and 200 units/ml streptomycin. Cells were plated into 75cm^2 tissue culture flasks and split 1:1 when confluent. Cells were characterised as mesothelial cells based on their morphology in culture. At confluence, mesothelial cells adopt a cobblestone appearance. Mouse mesothelial cells could not be characterised by standard cytokeratin expression immunohistochemically as mouse cytokeratin antibodies are not available. Cultures were used up to passage 3.

2.2 Cell proliferation assays

2.2.1 Assessment of DNA synthesis by measurement of ^{3}H-thymidine incorporation

To investigate the effect of test mediators including; porcine transforming growth factor-β_1 (TGF-β_1), TGF-β_2, TGF-β_3 (R&D Systems Europe, Abingdon, UK), thiaproline (Sigma Aldrich) and TGF-β neutralising antibodies (R&D Systems Europe and Cambridge Antibody Technology, Cambs, UK) on cell proliferation, a tritiated thymidine (^{3}H-TdR, Amersham, Buckinghamshire, UK) assay was used to measure DNA synthesis. Cells were plated into 96 well tissue culture plates in DMEM containing 1% (v/v) FBS (4000 cells / 100 μl / well). Only the central 10 x 6 wells of the plate were used to avoid edge artefact effects. The outer wells were filled with 100 μl of DMEM. After 24 hr the medium was removed and replaced with fresh DMEM containing 1% (v/v) FBS supplemented with the test mediator (100 μl / well) and ^{3}H-TdR (74 KBq/ml). After 24 hr in culture the cells were lysed by the addition of 10 μl of 10 M NaOH to each well and the plate frozen at -40°C prior to further analysis.

The lysed cells were thawed and the DNA harvested and washed on glass filter mats (ICN Biomedicals, Aurora, Ohio, USA) with a Micro96 cell harvester (Skatron, Lier, Norway). The filter for each well was shaken into 4 ml of Ecoscint A scintillation fluid (National Diagnostics, Atlanta, USA) contained in Pico 2000 polyethylene vials (Packard Instruments). The samples were read on a Minaxiβ liquid scintillation counter (Packard Instruments). Incorporated ^{3}H-TdR was measured in disintegrations per minute (d.p.m.) with the background count (scintillation fluid alone) subtracted from all values.

2.2.2 Substrate dependent proliferation

Coated tissue culture plates were used to study the influence of collagen on cell proliferation. Rat tail collagen type I (First Link, Birmingham, UK) and murine collagen type IV (Sigma Aldrich) were diluted in PBS to a range of concentrations between 0 – 10 μg/ml, and 50 μl of these solutions were applied to a 96 well plate. The plate was left open in a tissue culture hood overnight to dry. The following day the wells were washed with 100 μl PBS. To block non-specific binding of cells to any uncoated plastic on the bottom of the wells, 50 μl of 3% bovine serum albumin (BSA; w/v) in PBS was added to each well for 1 hr. The BSA solution was aspirated and cells plated at 4000 cells / 100μl / well. The rate of proliferation was assessed by the measurement of DNA synthesis, as detailed above (section 2.2.1).

2.3 Cytotoxicity assays

The proline analogue thiaproline was used in this study as an inhibitor of collagen synthesis. To ensure that thiaproline was being used at non-toxic concentrations, cell integrity and viability after thiaproline exposure was assessed using two standard biochemical markers of toxicity:

(i) cellular uptake of the supravital dye neutral red,

(ii) release of cytoplasmic lactate dehydrogenase (LDH).

2.3.1 Neutral red cytotoxicity assay

Neutral red is a cationic dye that readily diffuses across the plasma membrane of viable cells. The dye concentrates in the lysosomal matrix through electrostatic bonding to anionic sites. Disruption to the cell surface and the sensitive lysosomal membrane leads to decreased uptake and binding of the dye. This allows discrimination between viable and damaged cells.

Cells were plated in 96 well tissue culture plates in DMEM containing 1% (v/v) FBS (4000 cells / 100 μl / well) and incubated for 24 hr when experimenting on subconfluent cells, or allowed to grow to visual confluence (3 days). The culture medium was aspirated and groups of 6 wells supplemented with thiaproline (0 – 50 mM) in DMEM containing 1% (v/v) FBS.

After 24 hr the culture medium was removed and the cell layer washed with 100 µl of PBS. Cells were incubated with a 100 µl solution of neutral red (0.005% w/v in DMEM containing 1% (v/v) FBS) at 37°C for 3 hr. The first row received DMEM supplemented with 1% (v/v) FBS without neutral red to serve as a blank for the spectrophotometric analysis.

Following incubation, the neutral red solution was removed by gentle blotting over absorbent paper and the cells were washed with 2 x 100 µl of PBS to remove any excess dye. The cells were lightly fixed with 100 µl per well of an aqueous solution of formaldehyde (4% v/v) and calcium chloride (1% w/v). This was limited to 2 min to avoid damage to the lysosomes. The fixative was removed by blotting and the bound neutral red eluted from the cells by adding 200 µl of a solution of glacial acetic acid (1% v/v) and ethanol (50% v/v) to each well. To aid the elution of the dye into the acetic acid-ethanol solution, the 96 well plate was placed on an orbital shaker (Luckham, Sussex, England) at room temperature (RT) for 30 min. The absorbance per well was measured at 540 nm on a microplate photometer (Titertek Multiskan MC, ICN Flow, Buckinghamshire, UK) using the wells that did not receive neutral red treatment as blanks.

2.3.2 Measurement of lactate dehydrogenase release

Cellular release of lactate dehydrogenase (LDH) was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA) to determine the cytotoxic effects of thiaproline *in vitro*. LDH is a stable cytosolic enzyme (catalysing the interconversion of lactate and pyruvate) released upon cell lysis. Released LDH in culture supernatants was measured with a 30 min coupled enzymatic assay, which leads to the conversion of a tetrazolium salt into a red formazan product. The intensity of the colour is proportional to the number of lysed cells and is therefore an index of cytotoxicity.

Cells were seeded at 4,000 cells per well in 100 µl of DMEM containing 1% (v/v) FBS in a 96 well plate. The cells were grown to confluence and the medium replaced with thiaproline (0 – 40 mM) in DMEM containing 1% (v/v) FBS at 37°C for 24 hr.

The supernatant from each well (containing LDH release caused by the experimental treatment) was transferred to another 96 well plate, and 100 µl of lysis buffer (9% v/v Triton® X-100) was added to each well containing the intact cell layer and incubated at 37°C for 60 min. The plate was then centrifuged (250 x g, 4 min). Aliquots from all wells (50 µl) were transferred to a fresh 96 well plate and 50 µl of reconstituted substrate mix (lyophilised diaphorase, lactate and NAD^+ reconstituted with 1% BSA in PBS) was added to each well of the plate. The plate was wrapped in foil to protect it from light and incubated at RT for 30 min. A 50 µl volume of stop solution (1M acetic acid) was added to each well and the absorbance was read at 490 nm on a microplate photometer.

Cell death was expressed as a percentage of experimentally-induced cell LDH release over the maximum possible cell LDH release using the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Supernatant LDH content } (OD_{490})}{\text{Maximum (supernatant + lysed cell layer) LDH release } (OD_{490})} \times \frac{100}{1}$$

2.4 Measurement of procollagen production by High Performance Liquid Chromatography

Procollagen production by cultured cells was assessed by measuring hydroxyproline (hyp) in the ethanol-insoluble protein fraction of the cell monolayer and media compartments using a high performance liquid chromatography (HPLC) method developed in this laboratory (Campa *et al.*, 1990; McAnulty *et al.*, 1991).

Hyp represents approximately 12% of the primary amino acid sequence of procollagen (Laurent *et al.*, 1981) and is essential for the formation of the collagen triple helix. However, the amino acid is not at a significant level in other proteins with the exception of elastin (between 0.9 – 1.9% depending on species; Paz *et al.* 1982), complement protein C1q (4% hyp; Reid, 1979), acetylcholinesterase (5% hyp; Mays and Rosenberry, 1981) and surfactant apolipoprotein A (5.9% hyp; Hawgood, 1989). Mesothelioma cells in culture have not been shown to produce these proteins, and assuming that they do not, measuring the hyp content from cell cultures is taken to

represent procollagen production. However, some of the hyp measured may be from non-collagenous protein.

All water used for the preparation of HPLC buffers was purified and deionised using a Millipore Water Purification System (Millipore R010 followed by Milli-Q Plus; Millipore, Watford, UK). HPLC components and solvents used for the preparation of HPLC buffers and solutions were of HPLC grade and obtained from BDH-Merck.

2.4.1 Cell culture conditions

Cells from confluent cultures were trypsinised and counted (section 2.1), resuspended in DMEM containing 10% (v/v) FBS and seeded into 12 well tissue culture plates ($5x10^4$ cells / ml / well). For all collagen synthesis experiments, duplicate plates were prepared; one for hyp measurement and the other to obtain cell number. Both plates were treated identically.

When confluent, the culture medium in each well was removed and replaced with 1 ml of pre-incubation medium (DMEM containing 1% (v/v) FBS supplemented with 50 μg/ml ascorbic acid and 0.2 mM proline). After 24 hr the pre-incubation medium was removed and replaced with incubation medium consisting of test solutions in 1 ml of DMEM containing 1% (v/v) FBS supplemented with 50 μg/ml ascorbic acid and 0.2 mM proline. One plate containing cells exposed to fresh pre-incubation medium was used to obtain cell counts at the beginning of the experiment (t_0, 4 wells; section 2.4.2), and the remaining wells were stored at -40°C to determine the level of hyp at the start of the incubation period in the cell layer and medium.

After 24 hr of treatment with the incubation medium the plates designated for hyp analysis were stored at -40°C whilst cell counts were performed on the duplicate set of plates.

2.4.2 Assessment of cell number

The culture medium was removed from the plates for cell counts and each well was washed with 1 ml PBS to remove any remaining serum. Cells were incubated with 1 ml of trypsin (0.05%, w/v) / EDTA (0.02%, w/v) at 37°C for 4 min and cell

detachment confirmed by phase-contrast light microscopy. The cells were resuspended in the trypsin / EDTA solution and counted as described previously (section 2.1).

2.4.3 Cell harvesting

The plates were thawed and the cell layer in each well scraped into the medium using a cell scraper (Orange Scientific, Oxfordshire, UK). The contents of each culture well were aspirated and transferred to glass tubes. Each well was washed with a further 1 ml of PBS to remove any remaining protein, which was then transferred to the corresponding tube. Proteins were precipitated by the addition of absolute ethanol to a final concentration of 67% (v/v) at 4°C overnight.

2.4.4 Separation of ethanol-insoluble and ethanol-soluble protein fractions

Precipitated protein (ethanol-insoluble) was separated from free amino acids and small peptides (ethanol-soluble) by filtration onto a Durapore® membrane filter (filter type 0.45 μm HV, Millipore) using a vacuum filtration unit (Millipore). The proteins adhered to the filter were washed with 2 x 1.5 ml of 67% (v/v) ethanol. The filters were transferred to hydrolysis tubes and hydrolysed in 3 ml of 6 M HCl at 110°C for 16 hr. Hydrolysates were decolourised with approximately 30 mg of activated charcoal (BDH-Merck) and filtered through a membrane filter (filter type 0.65 μm DA, Millipore).

2.4.5 Derivatisation of samples

An aliquot (150 μl) of the decolourised hydrolysate was placed into a 1.5 ml microfuge tube and the sample dried under vacuum and heat on a Speed-Vac sample concentrator (Savant, Lutterworth, UK). The hyp content of the concentrated hydrolysate was isolated and measured by reverse-phase-HPLC following derivatisation with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) as described previously by Campa *et al.* (1990). NBD-Cl reacts with amino and imino (hydroxyproline and proline) acids to produce light absorbing reaction products. Imino acid reaction products exhibit strong light absorption between wavelengths of 450 – 550 nm, whereas amino acid products have limited absorbance in this range. Also, the reaction with imino acids occurs an order of magnitude faster than the

reaction with amino acids (Perrett, 1985). Therefore, the reaction with NBD-Cl and the measurement of absorbance between 450 – 550 nm gives a quantitative measurement of the amount of imino acid in the sample when compared to a standard containing a known concentration of hyp. Incubation times past 20 min were found to have no significant effect on the extent of derivatisation (Lindblad and Diegelmann, 1984).

Each dried hydrolysate sample was re-dissolved in 100 µl HPLC-grade deionised water, buffered with 100 µl of 0.4 M potassium tetraborate (pH 9.5) and reacted with 100 µl of 36 mM NBD-Cl in methanol. The samples were vortexed and incubated at 37°C for 20 min in the dark (NBD-Cl derivatised samples are light sensitive). To stop the reaction, the pH of each sample was lowered by the addition of 50 µl of 1.5 M HCl. To make the samples compatible with the initial HPLC running buffer, 150 µl of concentrated buffer A (167 mM sodium acetate in 26% acetonitrile v/v, pH 6.4) was added to each sample. Following vortexing and filtering (0.2 µm pore size syringe filter unit, Millipore, Watford, UK) samples were loaded onto a cooled autosampler (4°C, System Gold 507e, Beckman Coulter, High Wycombe, UK) from which a 100 µl aliquot was injected onto the HPLC column and eluted with an acetonitrile gradient.

2.4.6 Instrumentation and chromatographic conditions

Derivatised samples were separated on a Beckman System Gold HPLC apparatus (Beckman Coulter) with a reverse-phase cartridge column (LiChroCART LiChrospher, 250 mm length x 4 mm diameter, 5 µm particle size, 100RP-18) protected by a directly coupled precolumn (LiChrosorb, 4 x 4 mm, 5 µm particle size, 100RP-18). The column was continuously maintained at 40°C in a heated oven. Running buffers A (8% v/v acetonitrile and 50 mM sodium acetate, pH 6.4) and B (75% v/v acetonitrile) were freshly prepared and filtered (0.22 µm pore size type GV filter, Millipore) prior to use. The column was equilibrated in running buffer A for 40 minutes before being injected with the first sample. The first three samples measured on the column were hyp standard solutions (containing 50 pmol hyp) which were subsequently used for calibration.

NBD-Cl derivatives were eluted through the column in an acetonitrile gradient, generated by a shift in the relative proportions of the running buffers over time. The column running conditions are summarised in table 2.1. Post-column detection was achieved by monitoring absorbance at 495 nm using a flow-through variable wavelength monitor. The signal was processed for later quantification using an on-line chromatographic computing integrator (System Gold, Beckman Coulter). A defined peak corresponding to hyp eluted from the column approximately 5 min after sample injection. Total running and column regeneration time was 25 min per sample.

Column	LiChrospher, 100 RP-18, 250 x 4mm, 5μm	
Mobile phase buffers	A – aqueous acetonitrile (8%, v/v) 50 mM sodium acetate, pH 6.4 B – aqueous acetonitrile (75%, v/v)	
Column flow rate	1.00 ml/min	
Column temperature	40°C	
Wavelength for detection	495 nm	
Elution gradient	Time (min)	% Buffer B
	0	0
	5	5
	6	80
	12	80
	12.5	0
	25	0

Table 2.1 Chromatographic conditions and buffers for the separation of hydroxyproline by reverse-phase HPLC (modified from Campa *et al.*, 1990)

2.4.7 Quantification of hydroxyproline

Hyp content in each sample was determined by comparing the area of the hyp peak on the chromatogram generated to those obtained from the standard solutions, derivatised and separated under identical conditions. All values were corrected for the amount of hyp in the ethanol-insoluble protein fraction of the t_0 sample, representing hyp content in the cell layer and medium at the onset of the incubation period. The t_0 corrected hyp value represented the procollagen produced by the cells over the 24 hr incubation period. The data was corrected for cell number and expressed as nmol hyp / 10^6 cells / 24 hr. The calculations used for quantifying hyp are shown below:

$$\text{nmol hyp} = \frac{\text{acid hydrolysis volume (3000 µl)}}{\text{volume aliquot dried (150 µl)}} \times \frac{\text{500 µl reaction mix}}{\text{100 µl loaded on column}} \times \frac{\text{HPLC value}}{1000}$$

$$\text{To correct for cell number: } \frac{\text{nmol hyp}}{\text{cell number}} \times 10^6 = \text{nmol hyp / } 10^6 \text{ cells / 24 hr}$$

2.5 Assessment of non-collagen protein synthesis

The measurement of non-collagen protein synthesis was based on the uptake of tritiated phenylalanine into the ethanol-insoluble protein fraction. As the proportion of phenylalanine in procollagen is very low (0.8 – 1.2% depending on species; Miller and Gay, 1982) incorporated radiolabelled phenylalanine into protein can be used as an assessment of non-collagenous protein.

Cell culture conditions were similar to those described for the measurement of hydroxyproline (section 2.4.1), except for the addition of 74 KBq/well of L-[4-^{3}H-phenylalanine] (^{3}H-phe, Amersham) to the incubation medium. Aliquots (25 µl) of decolourised ethanol-insoluble protein hydrolysate (prepared as described in sections 2.4.2 – 2.4.4) were shaken into 4 ml of Ecoscint A scintillation fluid (National Diagnostics) contained in Pico 2000 polyethylene vials (Packard Instruments). Samples were read on a Minaxiβ liquid scintillation counter (Packard Instruments). The ^{3}H-phe content per well was measured in disintegrations per minute (d.p.m.) with the background count (scintillation fluid alone) subtracted from all values.

2.6 Transforming Growth Factor-β activity bioassay

Measurement of active and latent TGF-β_1, -β_2 and -β_3 production in MM conditioned media and the characterisation of TGF-β neutralising antibodies were performed using a transfected mink lung epithelial cell (MLEC) bioassay, kindly provided by Dr D. Rifkin, New York Medical Centre, USA. The cell line was maintained in culture in standard growth medium supplemented with 200 μg/ml gentamycin (Sigma Aldrich). The MLEC cells were stably transfected with a TGF-β sensitive p800neoLUC vector (Abe *et al.*, 1994). The vector contained a truncated 800 base pair fragment (-799 to +71) of the 5' end of the human plasminogen activator inhibitor-1 (PAI-I) gene fused to the firefly luciferase reporter gene (figure 2.1).

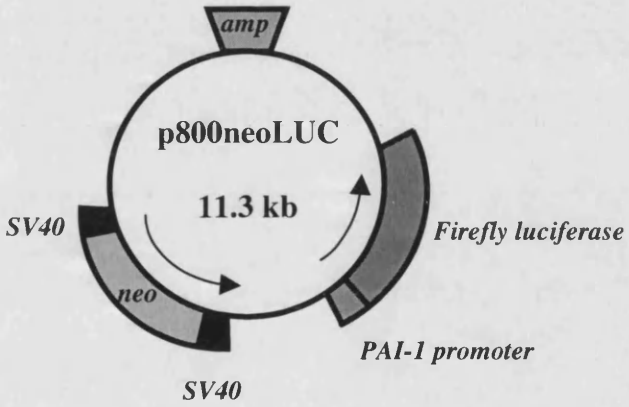


Figure 2.1 Map of p800neoLUC vector stably transfected into MLEC cell line. amp - ampicillin resistance gene, neo - neomycin resistance gene with flanking SV40 incorporation sequences (SV40)

Upon treatment with TGF-β, transfected MLEC cells were stimulated to produce intracellular luciferase via the PAI-1 promoter, the promoter being equally sensitive to all three mammalian isoforms of TGF-β. Following incubation, the cells were lysed and firefly luciferase activity measured via a bioluminescent reaction with beetle luciferase substrate (Promega). This transfected MLEC assay is extremely sensitive and covers a wide range of TGF-β concentrations (0.005 – 0.75 ng / ml), whilst being specific to TGF-β. Other modulators that may be present in biological fluids, such as EGF, FGF, IL-1α and PDGF do not activate the truncated PAI-1 construct (Abe *et al.*, 1994).

Confluent MLEC were trypsinised and counted (section 2.1) and resuspended in standard growth medium at 1.6 x 10^5 cells / ml. Cells were plated in 96 well tissue culture plates (100 µl / well) and incubated in 10% CO_2 for 3 hr at 37°C. Following cell attachment the medium was aspirated and 100 µl of TGF-β standards (recombinant TGF-β_1, -β_2, or -β_3 depending on the isoform being examined, in DMEM, 0 – 1 ng / ml range) and test samples were applied in triplicate to the cell layer. The plate was incubated in 10% CO_2 overnight (16 hr) at 37°C.

Following overnight incubation, the medium was aspirated and each well gently washed with 50 µl PBS. The cell layers were then lysed with 100 µl of 1 x cell lysis buffer (Roche Diagnostics) and incubated at RT for 20 min on an orbital shaker. Luciferase activity was assayed using the Luciferase Assay System (Promega). A 30 µl aliquot of the contents of each tissue culture plate well was transferred to an opaque 96 well Optiplate (Packard). Reconstituted beetle luciferase substrate (150 µl, Promega) was added to each test sample using a Tropix microplate luminometer (Perkin Elmer, Massachusetts, USA), which also measured the relative luminosity (relative light units, R.L.U.) of each sample over a 5 sec interval via Tropix WinGlow software (Perkin Elmer). A standard curve was plotted from the known concentrations of TGF-β (figure 2.2A) allowing conversion of R.L.U. to TGF-β conc. (ng/ml) and the quantity of active TGF-β present in the test samples calculated.

2.6.1 Assessment of active and total TGF-β in MM conditioned medium samples

Normal and malignant mesothelial cell cultures were grown to confluence in 75 cm^2 flasks, the monolayer washed with 10 ml PBS and then quiesced with serum free DMEM for 24 hr. The cell monolayer was washed with 10 ml PBS and replaced with 10 ml of serum free DMEM for 24 hr and then the medium removed and stored at -70°C. The remaining cell layer was trypsinised and cell number determined (section 2.1).

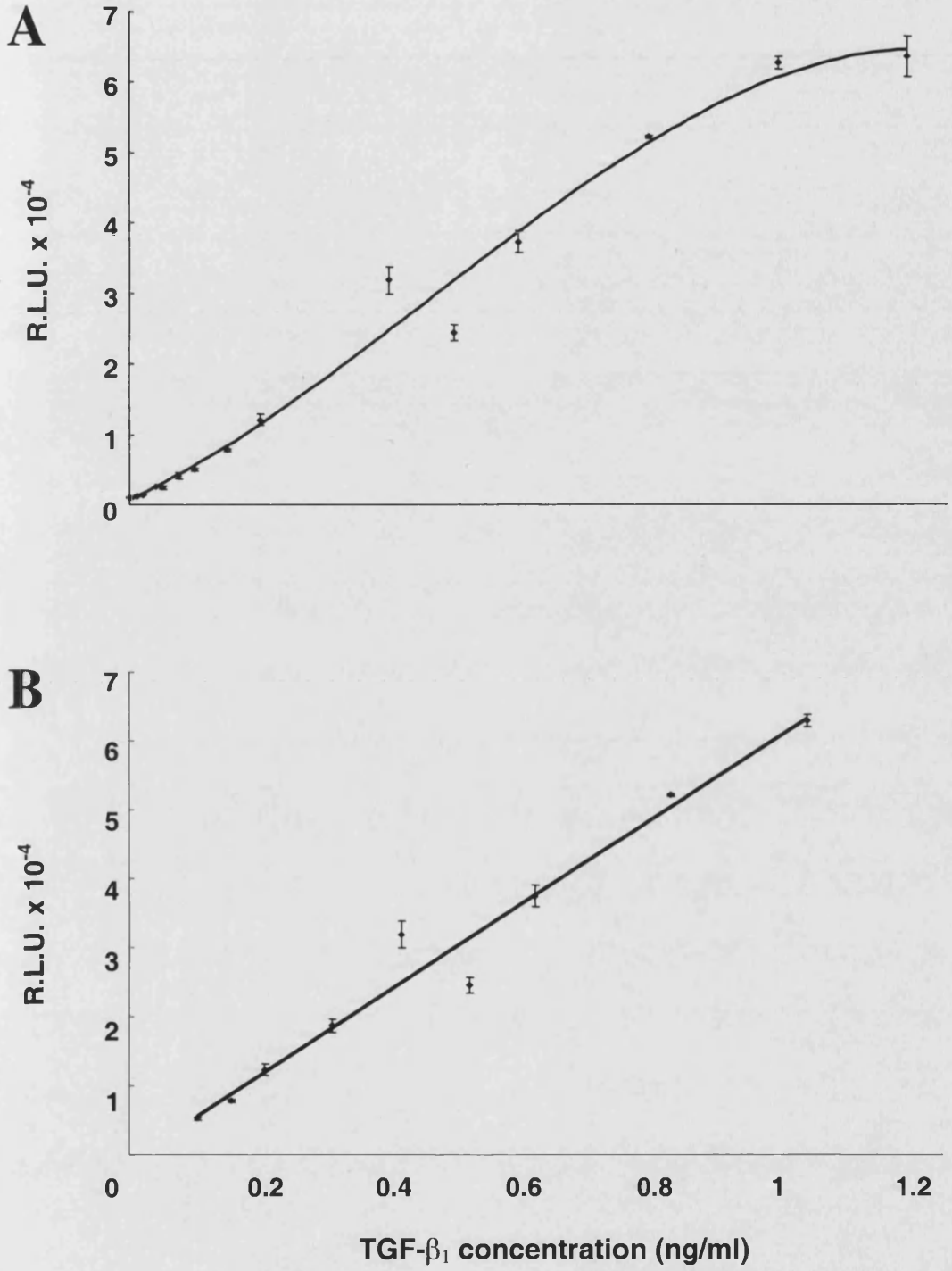


Figure 2.2 Dose-dependent induction of PAI-1-mediated luciferase expression (R.L.U.) by TGF-β_1. MLEC cells stably transfected with a PAI-1 / luciferase construct were incubated with increasing concentrations of TGF-β_1 for 16 hr and luciferase production measured via a bioluminescent reaction. **A** demonstrates a typical sigmoidal-shaped standard curve, **B** represents the linear portion of the standard curve from which TGF-β concentrations in test samples were calculated. The data are representative of three repeat experiments. Standard curves using TGF-β_2 and -β_3 had a similar appearance.

TGF-β activity in the test samples was assayed with the MLEC bioassay (section 2.6). Initial experiments were performed to ensure that the test sample R.L.U. values were within the linear range of the standard curve (figure 2.2B). Samples that gave higher R.L.U. values than the linear portion of the standard curve were appropriately diluted with serum free DMEM. Latent TGF-β (TGF-β:LAP) was activated by heating the samples at 80°C for 10 min in a waterbath (Gleizes *et al.*, 1997). A time course was performed to ensure that total activation was occurring at 10 min using two separate conditioned medium samples (figure 2.3). Activated samples were diluted accordingly with serum free DMEM and assayed. TGF-β production was represented as ng TGF-β / 10^6 cells / 24 hr.

2.6.2 Characterisation of TGF-β neutralising antibodies

The ability of specific TGF-β antibodies to neutralise TGF-β_1, -β_2 and -β_3 was assessed using the MLEC assay (section 2.6). Test and control antibodies (table 2.2) were incubated with 0.1 ng / ml of TGF-β_1, -β_2 and -β_3 for 30 min at 37°C, a separate standard curve constructed for each TGF-β isoform. The antibody/TGF-β solution was then assayed with the MLEC assay to determine the efficacy and specificity of the antibody. Antibodies were used at the concentrations recommended by the manufacturers.

2.6.3 Quantitation of TGF-β isoforms produced in normal and malignant mesothelial cell conditioned medium

To determine the amount of different active and latent TGF-β isoforms produced by normal and malignant cells, unheated and heated conditioned medium (section 2.6.1) were incubated with R&D Systems TGF-β neutralising antibodies and TGF-β activity measured. Conditioned medium was diluted with DMEM to a concentration of 0.1 ng / ml TGF-β and incubated with R&D Systems anti-TGF-β_1, anti-TGF-β_2, and Cambridge Antibody Technology pan-specific TGF-β antibodies with the appropriate control antibodies for 30 min at 37°C. TGF-β activity was measured using the MLEC assay (section 2.6) and the amount of TGF-β_1, -β_2 and -β_3 calculated by comparing the different antibody treatment groups to each other and the antibody-free samples. TGF-β isoform production was represented as ng TGF-β / 10^6 cells / 24 hr.

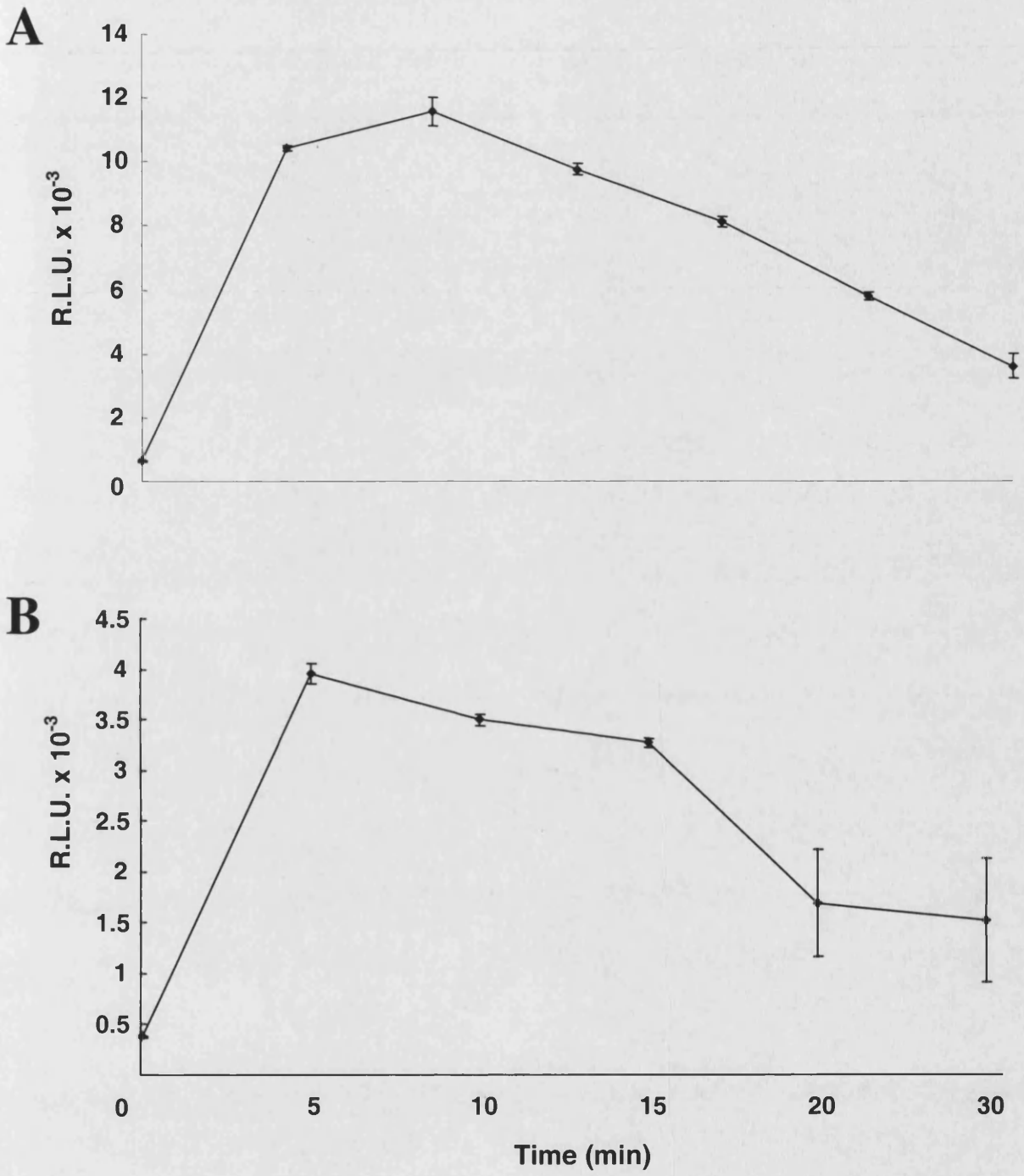


Figure 2.3 Heating time course of AB1 and AC29 conditioned medium. Conditioned medium harvested from AB1 (**A**) and AC29 (**B**) cell monolayers were collected and heated at 80°C to activate latent TGF-β. TGF-β activity was assessed via luciferase production using the MLEC bioassay. This data suggested the optimal activation of latent TGF-β occurred between 5 and 10 min at 80°C, after which the amount of retrievable TGF-β decreased. The data are representative of three repeat experiments.

Antibody / concentration used	Control antibody
Chicken anti-human TGF-β_1 (R&D Systems) **100 ng / ml**	Chicken globulin (Jackson ImmunoResearch, Pennsylvania, USA) **100 ng / ml**
Goat anti-human TGF-β_2 (R&D Systems) **100 ng / ml**	Normal goat IgG (R&D Systems) **100 μg / ml**
Goat anti-worm TGF-β_3 (R&D Systems) **100 μg / ml**	Normal goat IgG (R&D Systems) **100 μg / ml**
anti-TGF-β_1, -β_2, -β_3 murine IgG1 (R&D Systems) **100 μg / ml**	irrelevant murine IgG1 (R&D Systems) **100 μg / ml**
anti-TGF-β_1 human IgG4 (Cambridge Antibody Technology) **100 μg / ml**	irrelevant human IgG4 (Cambridge Antibody Technology) **100 μg / ml**
Acidified anti-TGF-β_1 human IgG4 (Cambridge Antibody Technology) **150 ng / ml**	Acidified irrelevant human IgG4 (Cambridge Antibody Technology) **150 ng / ml**
anti-TGF-β_2 human IgG4 (Cambridge Antibody Technology) **100 μg / ml**	irrelevant human IgG4 (Cambridge Antibody Technology) **100 μg / ml**
anti-TGF-β_1, -β_2, -β_3 murine IgG1 (Cambridge Antibody Technology) **100 μg / ml**	irrelevant murine IgG1 (Cambridge Antibody Technology) **100 μg / ml**

Table 2.2 Concentration of R&D Systems and Cambridge Antibody Technology control and TGF-β antibodies used for *in vitro* characterisation

2.7 Animal models of mesothelioma

Tumour growth studies *in vivo* were performed in female 8 week-old BALB/c and CBA mice (Harlan). The MM cell lines AB1 and AB22 were derived in BALB/c mice and AC29 cells in a CBA mouse. Introducing these cell lines back into the same strain of mouse does not initiate a host response. Female mice were used as this sex has a more constant body weight from 8 weeks of age upwards compared to the males, allowing the maintenance of an even dosing of thiaproline and TGF-β antibodies. Two murine models of mesothelioma were compared to determine the most consistent and reproducible method of tumour growth.

2.7.1 Intraperitoneal model of mesothelioma

Two groups of mice were injected intraperitoneally with 10^6 syngeneic MM cells in 100 µl of serum free DMEM and a further control group received no tumour cells (Davis *et al.*, 1992). After 18 days of thiaproline treatment (section 2.7.3), animals were sacrificed by cervical dislocation, a midline laparotomy performed and the abdomen contents examined. The nodular nature of the tumour scattered around the mesentery made dissection and separation from the host impractical. Thus, abdominal weight was chosen as a measure of tumour growth and the entire abdominal contents removed, weighed on a microbalance (Mettler, London, UK) and snap frozen in liquid nitrogen.

2.7.2 Subcutaneous model of mesothelioma

Mice were anaesthetised in a gas box with 4% halothane (Ohmeda, BOC gases, London, UK), 1.5 l/min oxygen and 1.5 l/min nitrogen (BOC gases) until unconscious. The hind flanks were shaved and both sides injected subcutaneously above the thigh with 10^6 syngeneic MM cells in 100 µl of serum free DMEM (figure 2.4). In pilot studies the two largest dimensions of the tumour were measured externally with microcallipers, and used to calculate tumour area. At 10 and 18 days (thiaproline treatment, section 2.7.3) and 15 and 19 days (TGF-β neutralising antibody studies, section 2.7.4) after injection of tumour cells, animals were sacrificed by cervical dislocation and the subcutaneous tumours surgically removed. The tumour from one flank was weighed on a microbalance and immersed in 4% paraformaldehyde (Sigma Aldrich). These tumours were paraffin embedded for histology (section 2.9). The

tumour from the other flank was weighed on a microbalance and snap frozen in liquid nitrogen. These tumours were transferred to a -70°C freezer for subsequent collagen analysis (section 2.7.5).

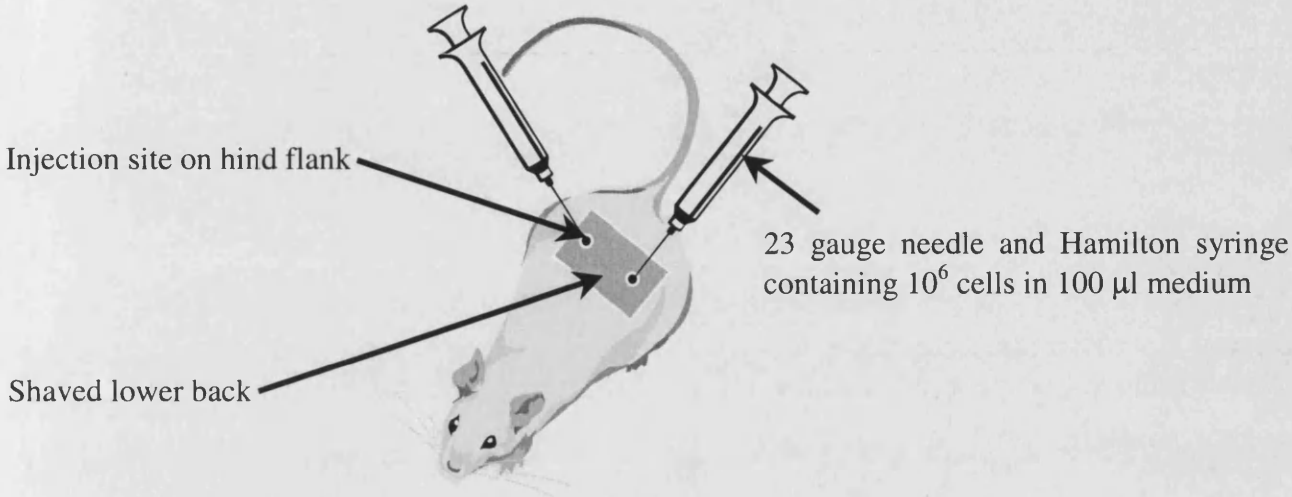


Figure 2.4 Subcutaneous injection of syngeneic cells into hind flanks of 8 week-old female mouse

2.7.3 Thiaproline in vivo studies

From day 1, one group of injected animals received thiaproline dissolved in their drinking water at an approximate dose of 100 mg/kg/day. The control injected group received normal drinking water. The thiaproline-treated water was prepared fresh daily and water intake measured by graduations on the water bottles. The mice were fed mouse chow *ad libitum* and were monitored daily for signs of ill health. The mice were weighed at regular intervals (approximately every three days) throughout the experiment.

To measure circulating levels of thiaproline in blood plasma, a midline laparotomy was performed and the abdominal contents moved to one side. Blood was harvested from the inferior vena cava via a heparinised 25 gauge needle and 1 ml syringe (heparin from CP Pharmaceuticals, Wrexham, UK) and stored at 4°C in a heparinised

eppendorf tube (Eppendorf, Hamburg, Germany) to be later analysed for thiaproline levels (section 2.8).

2.7.4 TGF-β neutralising antibody in vivo studies

Monoclonal TGF-β antibodies were generously supplied for *in vivo* studies by Cambridge Antibody Technology: CAT-192 (anti-TGF-β_1 human IgG4), CAT-152 (anti-TGF-β_2 human IgG4), CAT-001 (irrelevant human IgG4), 1D11 (anti-TGF-β_1, -β_2, -β_3 murine IgG1) and 13C4 (irrelevant murine IgG1).

Groups of 5-10 animals were separated into treatment groups; anti-TGF-β antibody treated (5 mg/kg in 100 µl PBS), control antibody treated (5 mg/kg in 100 µl PBS) and PBS control treated (100 µl). Prior to injection of tumour cells (24 hr), animals were injected intraperitoneally with the treatment antibody / control and subsequently at 3 day intervals until the end of the experiment using a 21 gauge needle and 1 ml syringe. Animals had free access to food and water.

2.7.5 Measurement of tumour collagen content by High Performance Liquid Chromatography

Frozen tumours were transferred to a ceramic mortar on ice and kept cool by the addition of liquid nitrogen. The tumour was crushed to a fine powder using a ceramic pestle. The powdered tumour tissue was hydrolysed in 2 ml of 6 M HCl at 110°C for 16 hr. Hydrolysates were decolourised with approximately 30 mg of activated charcoal (BDH-Merck) and filtered through a 0.65 µm pore DA filter (Millipore, UK). A 10 µl aliquot of decolourised sample was diluted 1:1000 times in deionised H_2O. A 200 µl aliquot of this diluted sample was dried to remove the acid, derivatised, and the hyp content quantified as described in sections 2.4.5 – 2.4.7. The data was corrected for the aliquot weight and was expressed as nmol hyp/ tumour.

2.8 High Performance Liquid Chromatography analysis of thiaproline in blood plasma

In order to determine the circulating blood plasma levels of thiaproline in treated versus non-treated mice, HPLC analysis was employed following the method of Lankelma *et al.* (1981).

2.8.1 Sample preparation

Heparinised mouse blood (section 2.7.3) was transferred to a 15 ml polypropylene tube and centrifuged at 2000 x g for 5 min to give a plasma supernatant. The supernatant was transferred to a fresh 15 ml polypropylene tube and deproteinised with 2 M perchloric acid (BDH-Merck) in a 4:1 ratio respectively. The solution was centrifuged at 2000 x g for 5 min to pellet the precipitated protein. In order to refine the detection method, human blood plasma was prepared by the same method above from a 50 ml human blood sample. A 50 µl aliquot of supernatant was injected onto the column and the rest stored at -70°C.

2.8.2 Instrumentation and chromatographic conditions

Deproteinised samples were separated on a Beckman System Gold HPLC apparatus (Beckman Coulter) with a microparticulate strong cation exchange column (Partisil SCX, Whatman, New Jersey, USA). The column dimensions were 250 mm length x 4.6 mm internal diameter and 10 µm particle size. The column was kept at ambient temperature. Running buffer consisting of 90% v/v 0.01 M phosphoric acid, pH 2.2 and 10% v/v acetonitrile was freshly prepared and filtered (0.22 µm pore size type GV filter, Millipore) prior to use. The column was equilibrated in running buffer for 40 min before applying the first sample. The first six samples injected onto the column were standard solutions, consisting of duplicates of thiaproline spiked human blood plasma at concentrations of 0.01 mM, 0.1 mM and 1 mM, which were used for calibration. Plasma samples were eluted through the column in a continuous flow of running buffer (1 ml / min).

Post-column detection was achieved by monitoring absorbance at 205 nm using a flow-through variable wavelength monitor. The signal was processed for later quantification using an on-line chromatographic computing integrator (System Gold, Beckman Coulter). A peak corresponding to thiaproline eluted from the column approximately 8 min after sample injection. Total running and column regeneration time amounted to 25 min per sample.

2.8.3 Quantification of thiaproline

Thiaproline content in each sample was determined by comparing the area of the thiaproline peak on the chromatogram to those generated from the standard solutions, separated under identical conditions. The data was expressed as circulating thiaproline (mM) in blood plasma.

2.9 Histology

2.9.1 Tumour tissue processing

Surgically removed tumours (section 2.7.2) were fixed in 4% paraformaldehyde buffered in PBS, pH 7.4, at 4°C overnight. The tissue was washed twice in PBS for 30 min and placed in 15% sucrose in PBS at 4°C overnight. The tissue was then washed in PBS for 30 min, 50% ethanol for 30 min and in 70% ethanol at 4°C overnight before being further processed and wax embedded (table 2.3) using a Leica TP 1050 vacuum tissue processor (Leica, Nussloch, Germany).

2.9.2 Tissue section preparation

Wax blocks were placed on ice prior to cutting. Tissue sections (5 μm) were prepared using a retraction microtome (Shandon, Runcorn, UK) and placed on poly-L-lysine-coated slides (BDH-Merck). The sections were air dried overnight before staining. Tissue sections were dewaxed in xylene and rehydrated in decreasing concentrations of industrial methylated spirit (IMS; table 2.4) using an automated stainer (Sakura DRS-601 Diversified Stainer, Bayer, Newbury, UK) prior to staining. Following staining, sections were dehydrated through an increasing concentration of IMS (table 2.5) using an automated stainer and mounted using an automatic coverslipping machine (Sakura Coveraid, Bayer).

Reagent	Time (hr:min)	Temp (°C)
70% industrial methylated spirit (IMS)	2:00	37
90% IMS	1:30	Ambient
90% IMS	1:30	Ambient
Absolute ethanol	1:00	Ambient
Absolute ethanol	1:15	Ambient
Absolute ethanol	1:15	Ambient
Absolute ethanol	1:15	Ambient
Xylene	1:00	Ambient
Xylene	1:00	Ambient
Xylene	1:00	Ambient
Paraffin Wax	1:00	60
Paraffin Wax	1:00	60
Paraffin Wax	1:00	60

Table 2.3 Processing schedule of Leica TP1050 Vacuum Tissue Processor to wax embed tumour samples

Reagent	Time (min)
Xylene	3
Xylene	3
100% IMS	2
90% IMS	2
70% IMS	2
50% IMS	2
Deionised Water	1

Table 2.4 Dewaxing and rehydrating schedule for paraffin sections

Reagent	Time (min)
Deionised Water	1
50% IMS	1
70% IMS	1
90% IMS	1
100% IMS	1
100% IMS	2
Xylene	1
Xylene	1
Xylene	2

Table 2.5 Dehydrating schedule for stained sections

2.9.3 Modified Martius Scarlet Blue stain

Modified Martius Scarlet Blue is a trichrome stain allowing the differentiation of compartments within a tissue section through their varying permeabilities to different dyes. Dewaxed and rehydrated tumour sections were stained using an automated slide stainer. Sections were treated in Lugol's iodine (BDH-Merck) for 5 min to enhance subsequent staining. Sections were decolourised in sodium thiosulphate (BDH-Merck) for 3 min and then washed in tap water for 1 min. The nuclei were stained using the celestine blue / haematoxylin sequence. The slides were immersed in celestine blue (Lamb, London, UK) for 10 min and washed in tap water for 1 min followed by deionised water for 30 sec. Slides were then stained in haematoxylin (BDH-Merck) for 5 min and sections differentiated in tap water for 30 sec, acid alcohol (1% HCl in 70% ethanol) for 20 sec, and two washes of tap water for 30 sec and 2 min. Erythrocytes in the section were stained in aqueous Orange G (Lamb) for 8 min, the section then differentiated in deionised water for 5 sec. Fibrin within the sections was stained in red mix (0.5% ponceax de xylidine and 0.5% acid fuchsin in 1% glacial acetic acid, BDH-Merck) for 7 min, and the sections differentiated in deionised water for 20 sec, phosphotungstic acid (1% in deionised water, BDH-Merck) for 30 sec followed by deionised water for 20 sec. Collagen present in the

sections was stained with Chicago sky blue (1% in glacial acetic acid, Sigma Aldrich) for 5 min and then differentiated in 1% acetic acid. The sections were washed, dehydrated, cleared and coverslipped (section 2.9.2). Nuclei stained dark blue/black, erythrocytes orange/yellow, fibrin scarlet and collagen bright blue.

2.9.4 Reticulin stain

Dewaxed and rehydrated tumour sections were oxidised in acidified potassium permanganate (BDH- Merck) for 3 min and then washed in tap water. The sections were bleached in 1% oxalic acid for 2 min and then washed in tap water followed by deionised water. The slides were sensitised in 2.5% ferric ammonium sulphate (BDH-Merck) for 10 min then rinsed well in deionised water. The sections were impregnated with silver solution (ammonia added dropwise to 5 ml 10.2% aqueous silver nitrate (BDH- Merck) until precipitate that first formed just dissolved. Sodium hydroxide solution (3.1%, 5 ml) was added and ammonia added dropwise until the precipitate dissolved and the volume made up to 50 ml with deionised water) for 30 sec. The sections were washed in deionised water and reduced in 10% aqueous formalin (BDH- Merck). The slides were washed in tap water, toned in 0.1% gold chloride for 1 min, then washed again in tap water and fixed in 5% sodium thiosulphate before being washed and counter stained in 0.2% neutral red solution (BDH-Merck). The slides were washed, dehydrated, cleared and coverslipped (section 2.9.2). Reticulin was evident as black fibres within the sections.

2.9.5 Haematoxylin and eosin stain

Dewaxed and rehydrated tumour sections were stained using an automated slide stainer. Slides were stained in Gill's haematoxylin (Shandon) for 5 min and then washed in tap water for 20 sec. Sections were differentiated in 1 % acid alcohol (1% HCl in 70% ethanol) for 8 sec and then washed in tap water for 2.5 min. The slides were then stained in 1% eosin (Shandon) for 6 min. The sections were washed, dehydrated, cleared and coverslipped (section 2.9.2). Nuclei stained dark blue/black, cytoplasm varying shades of pink, erythrocytes orange/red, and fibrin deep pink.

2.9.6 Immunohistochemistry

Immunohistochemical techniques allow the identification and localisation of cellular and tissue components through the binding of specific antibodies raised against them.

The site of antigen-antibody interaction is visualised either by direct antibody labelling (such as a fluorescent tag) or through the use of a secondary labelling method.
Dewaxed and rehydrated tumour sections were permeabilised on ice for 5 min with PBS, pH 7.0, containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), 300 mM sucrose, 50 mM sodium chloride, 3 mM magnesium chloride and 0.5% Triton X-100 (Sigma Aldrich) to unmask the epitopic sites. The slides were washed in three changes of PBS for 15 min on a stirrer. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Sigma Aldrich) in a humidified chamber for 30 min. The slides were washed in three changes of PBS for 15 min followed by incubation with serum of the host secondary antibody (1:25 dilution) in a humidified chamber for 20 min to reduce non-specific antibody binding. The serum was drained off and initially, to determine the optimal concentration of primary antibody to use (table 2.6), the tissue was incubated with dilutions of the primary antibody (0.1 – 20 μg / ml in PBS) in a humidified chamber overnight at 4°C. Once a suitable dilution had been determined for each antibody this was subsequently used, with an isotype control antibody at the same concentration (table 2.6).

The slides were washed in three changes of PBS for 15 min and sections incubated with the appropriate secondary antibody (1:200 dilution in PBS, table 2.6) for 1 hr in a humidified chamber at RT. The sections were washed in PBS three times and incubated with a 1:200 dilution of horseradish-peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark) in PBS for 30 min. The slides were washed for 15 min in three changes of PBS and the peroxidase activity visualised using 3,3'diaminobenzidine (DAB) substrate for 10 min following the manufacturer's protocol (DAB substrate kit for peroxidase, Vector Laboratories, CA, USA). The sections were washed in deionised water, counterstained with 1% eosin, washed, dehydrated and coverslipped (section 2.9.2). Areas of positive reactivity demonstrated brown staining.

Primary antibody / optimal concentration	Control antibody	Secondary Antibody	Blocking serum
Rabbit anti-mouse Collagen Type I (Chemicon, CA, USA) **2.5 μg / ml**	Rabbit IgG (Dako)	Goat anti-rabbit biotin-conjugated (Dako)	Goat (Dako)
Rabbit anti-human Collagen type III (Chemicon) **10 μg / ml**	Rabbit IgG (Dako)	Goat anti-rabbit biotin-conjugated (Dako)	Goat (Dako)
Rabbit anti-human Collagen type IV (Chemicon) **7.5 μg / ml**	Rabbit IgG (Dako)	Goat anti-rabbit biotin-conjugated (Dako)	Goat (Dako)
Chicken anti-human TGF-β_1 (R&D Systems) **2 ng / ml**	Chicken IgG (Jackson ImmunoResearch)	Goat anti-chicken biotin-conjugated (Dako)	Goat (Dako)

Table 2.6 Primary / control antibodies, blocking serum and secondary antibodies used for TGF-β_1 and collagen type I, type III and type IV immunohistochemistry

2.9.7 Microscopy

Sections were examined using an Olympus BX40 microscope (Olympus Optical Co.) and imaged using a JVC KY-F55B colour video camera (Victor Co., Tokyo, Japan) in conjunction with Zeiss KS300 imaging software (Carl Zeiss, Welwyn Garden City, UK).

2.10 Statistical analysis

All cell data were expressed as mean ± standard error of the mean (SEM) for 6 replicates. For single group comparisons statistical analysis was performed using an unpaired Student's t-test. Multiple group comparisons were performed using ANOVA followed by an ad hoc unpaired Student's t-test. Due to unequal variances between the different tumour groups, statistical analysis comparing the control group to the treated groups was performed using a Mann-Whitney U test. The data was shown as a spread of tumour weight around the median. A p value less than 0.05 was considered statistically significant.

Chapter Three

Characterisation of normal and malignant mesothelial cell lines

3.1 Introduction

There is a growing body of evidence suggesting that the ECM, particularly collagen, may be important in tumour growth. It is generally believed that ECM protein-producing stromal cells, not the tumour cells, are the major contributors to the matrix of fibrous tumours (Kauppila *et al.,* 1998, Dahlman *et al.,* 2000, Yen *et al.,* 2002). However, previous experiments in this laboratory have shown that MM cells in culture synthesise collagen (a key ECM component) at rates three to four times greater than normal mesothelial cells, comparable to that of lung fibroblasts, the predominant collagen producing cells in the lung. The role of ECM proteins in MM have not been fully elucidated. MM cells also secrete TGF-β, a key regulator of cell growth and ECM / collagen production, at elevated levels compared to normal mesothelial cells and other tumour types (Fitzpatrick *et al.*, 1994, Maeda *et al.*, 1994). Additionally, different MM morphologies have been shown to affect disease progression, fibroblast-like cell morphology associated with a worse prognosis (Fusco *et al.,* 1993). The development of suitable panels of human and murine MM tumour cell lines in conjunction with animal models has enabled the biology of MM to be investigated in more depth (Manning *et al.,* 1991, Davis *et al.,* 1992). This initial chapter sought to characterise a series of murine and human normal and malignant cell lines. Specifically, to;

1. establish the morphology of the cell lines,
2. quantitate the amount of active and latent TGF-β produced,
3. assess the ability of exogenous TGF-β to induce procollagen production,
4. and to determine whether any correlation between cell morphology and procollagen / TGF-β production was evident.

3.2 Malignant mesothelioma cell line morphology

Murine (figure 3.1) and human (figure 3.2) cell lines were viewed under a light microscope at approximately 50% confluence and when the cells were fully confluent. All malignant cell lines exhibited loss of contact inhibition, continuing to grow by piling on top of the cell monolayer. Murine and human cell lines were heterogeneous in morphology, both species exhibiting epithelial and fibroblast-like cell lines. The mouse MM cell line AC29 (figure 3.1) was biphasic, containing epithelial and fibroblastic type cells. Morphologically, the most similar malignant cell line to normal

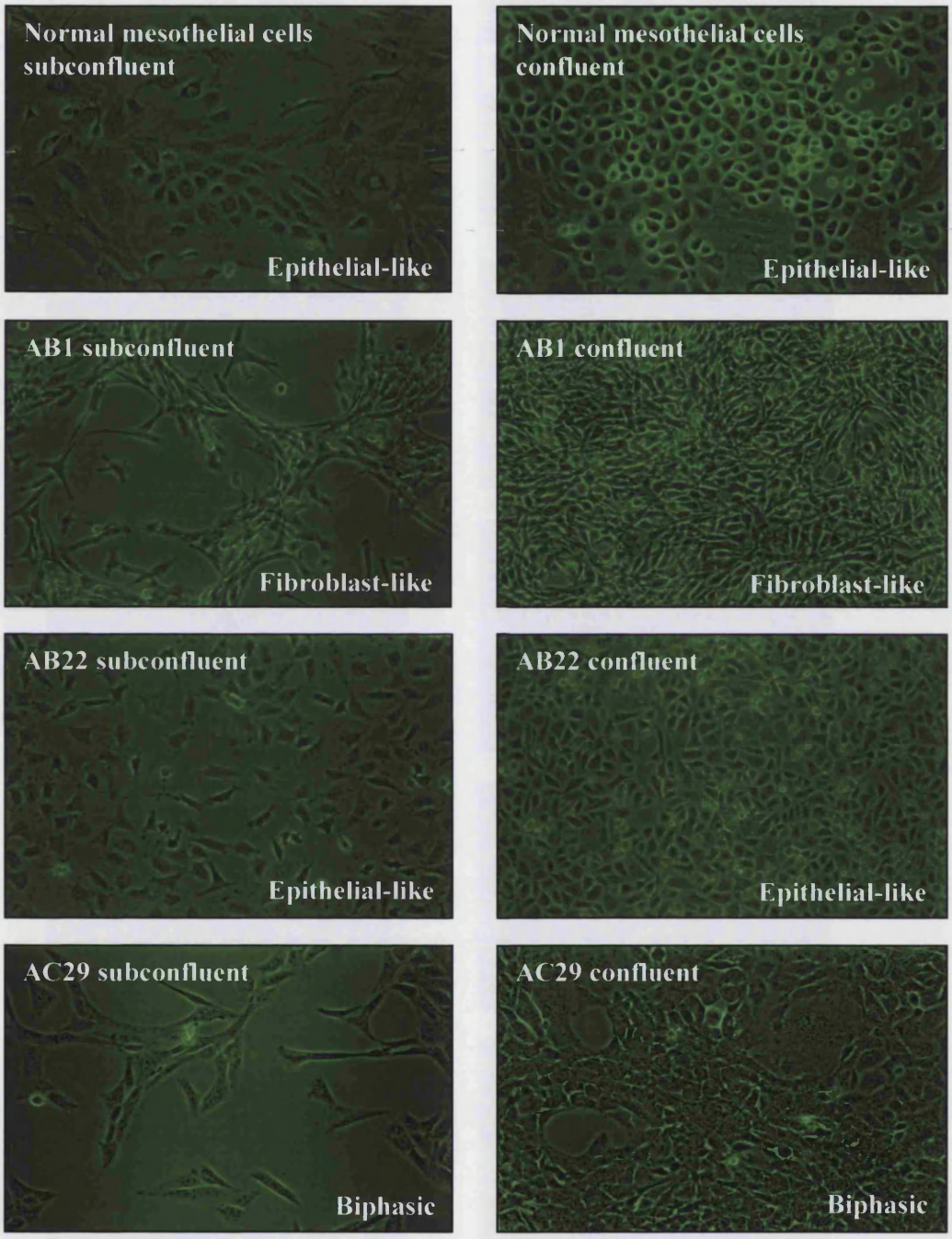


Figure 3.1 Murine normal and malignant mesothelial cell line morphology. Images of subconfluent and confluent murine mesothelial and MM cell lines demonstrate heterogeneity in cell appearance. Magnification 200x.

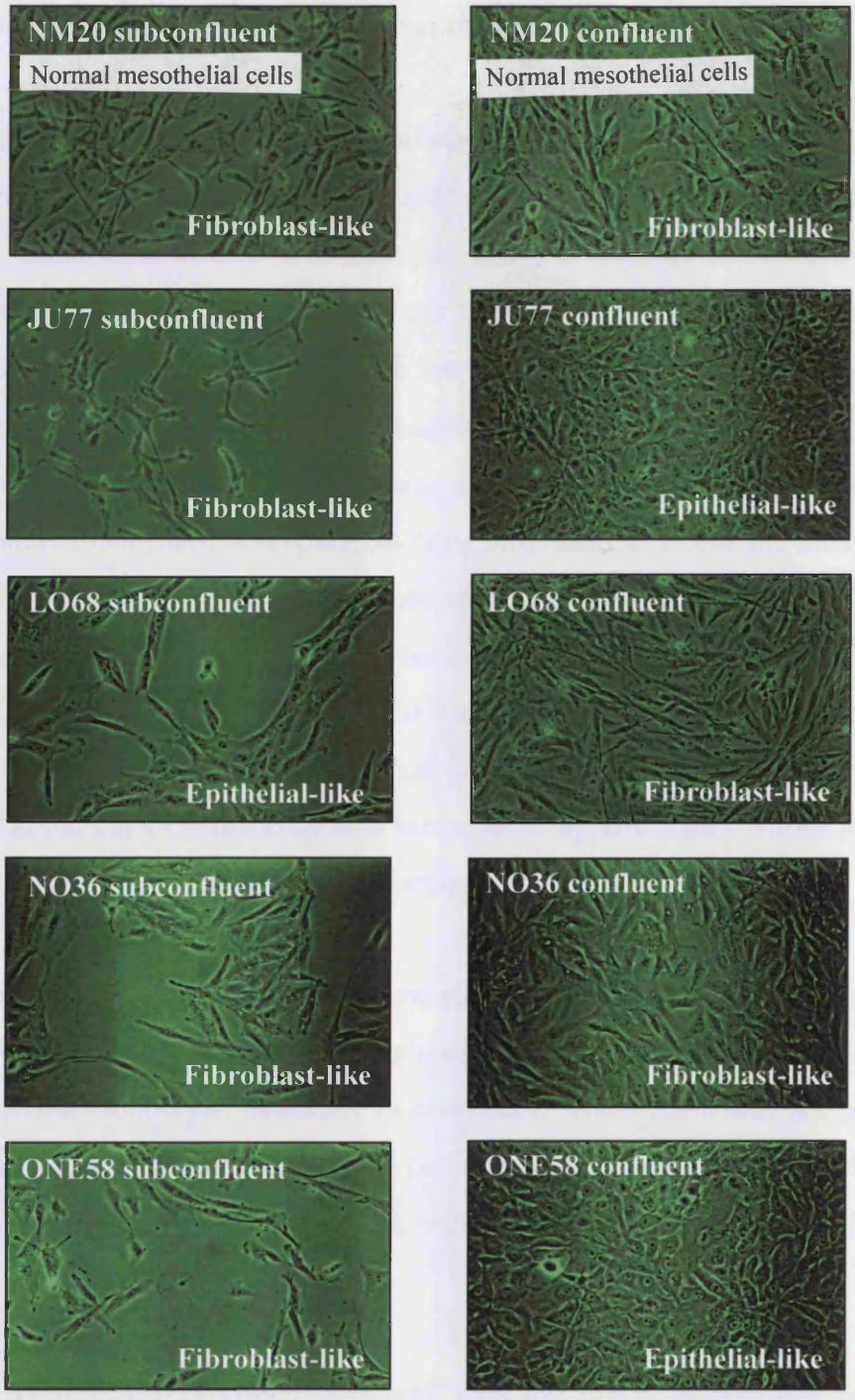


Figure 3.2 Human normal and malignant mesothelial cell line morphology. Subconfluent and confluent human mesothelial and MM cell cultures appeared heterogeneous in morphology. Magnification 200x.

primary cultured murine mesothelial cells was AB22, which had a characteristic epithelial 'cobblestone' appearance at confluence (figure 3.1). The most similar to NM20 (normal human mesothelial cell) was the malignant line LO68 (figure 3.2).

3.3 Malignant mesothelioma cell line endogenous TGF-β production

The amount of active, latent and total (active + latent) TGF-β produced by the cell lines into conditioned media was measured using a bioassay sensitive to all three mammalian isoforms of TGF-β. All of the cell lines examined expressed detectable TGF-β bioactivity (figure 3.3), although heterogeneity was seen between cell lines. Comparatively, similar trends were observed in active, latent and total TGF-β production between cell lines. AC29 was consistently the highest producer in the murine panel of cell lines. Compared to non-malignant CBA cells the malignant cell line AC29 produced a significantly higher amount of TGF-β (increases in AC29 of approximately 15-fold active, 17-fold latent and 17-fold total TGF-β production compared to non-malignant control, $p<0.001$ in each case). The murine MM cell line AB22 was the lowest producer of TGF-β, although still significantly elevated compared to the CBA control cells with increases of approximately 3-fold active, 1.6-fold latent and 1.7-fold total TGF-β production, $p<0.001$ in each case.

TGF-β production by the human normal mesothelial cell line NM20 was unable to be quantitated as during the 24 hr serum free quiescence period cells would round up and detach from the monolayer, indicative of cell death. Therefore it was not possible to compare TGF-β production in the human panel of MM cell lines with normal cells. In the human panel of cells, NO36 was the highest producer of TGF-β, LO68 was the lowest.

The majority of TGF-β produced by the cell lines was latent (approximate percentage of total TGF-β production that was latent: CBA normal mesothelial cell control 94%, AB1 97%, AB22 87%, AC29 95%, JU77 63%, LO68 72% and NO36 86%). The exception was the human cell line ONE58 that had a latent TGF-β production of approximately 5% of the total.

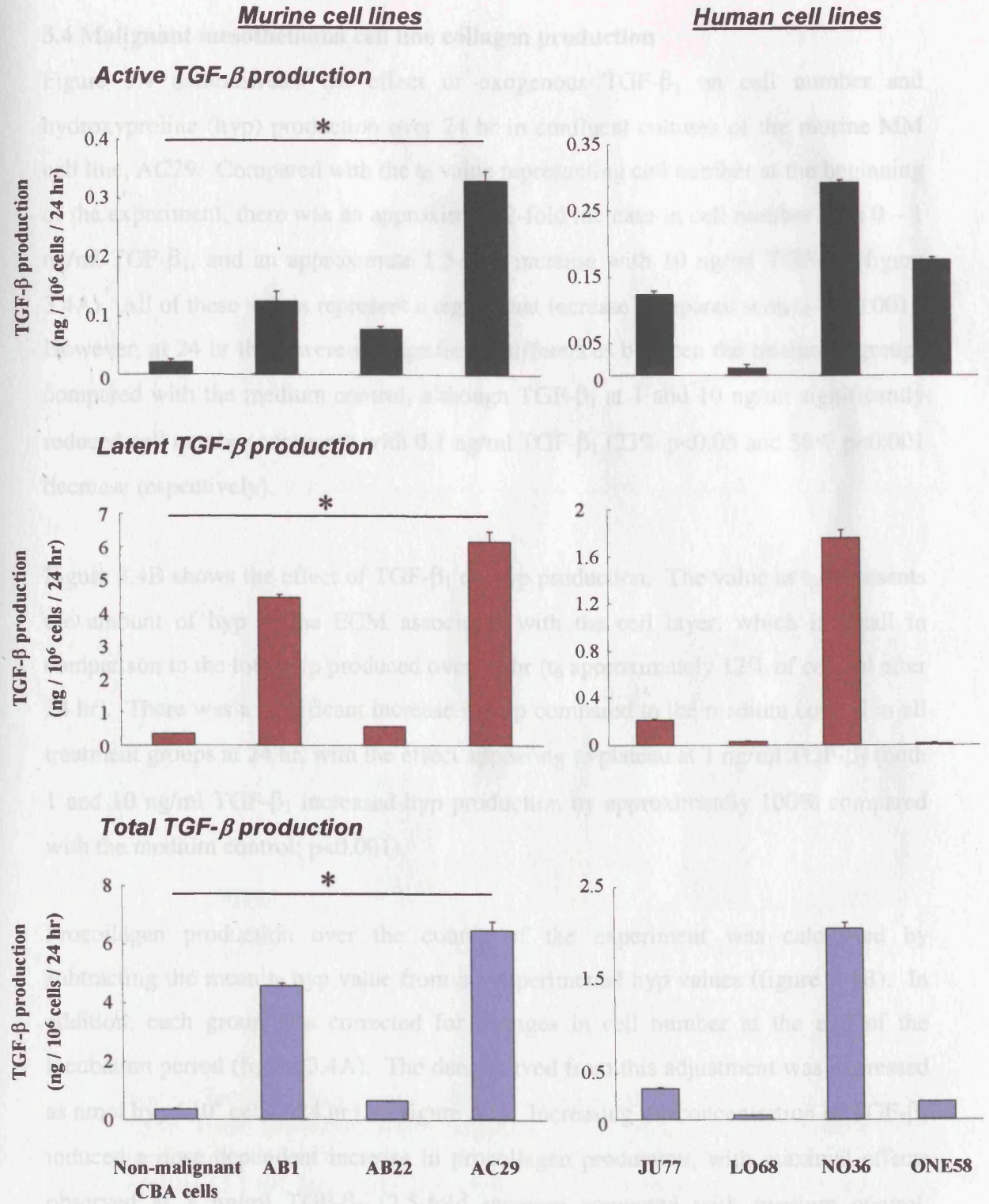


Figure 3.3 Active, latent and total TGF-β production by normal and malignant cell lines. Conditioned media harvested from normal and malignant cell monolayers were assayed for active and latent TGF-β (section 2.6.1). The results are representative of three sets of conditioned media each assayed at least twice. Each bar represents the mean ± SEM for six replicate wells. *$p<0.001$ for AC29 TGF-β production compared to CBA non-malignant control mesothelial cells.

3.4 Malignant mesothelioma cell line collagen production

Figure 3.4 demonstrates the effect of exogenous TGF-β_1 on cell number and hydroxyproline (hyp) production over 24 hr in confluent cultures of the murine MM cell line, AC29. Compared with the t_0 value representing cell number at the beginning of the experiment, there was an approximate 2-fold increase in cell number with 0 – 1 ng/ml TGF-β_1, and an approximate 1.5-fold increase with 10 ng/ml TGF-β_1 (figure 3.4A). All of these values represent a significant increase compared with t_0 ($p<0.001$). However, at 24 hr there were no significant differences between the treatment groups compared with the medium control, although TGF-β_1 at 1 and 10 ng/ml significantly reduced cell number compared with 0.1 ng/ml TGF-β_1 (23% $p<0.05$ and 38% $p<0.001$ decrease respectively).

Figure 3.4B shows the effect of TGF-β_1 on hyp production. The value at t_0 represents the amount of hyp in the ECM associated with the cell layer, which is small in comparison to the total hyp produced over 24 hr (t_0 approximately 12% of control after 24 hr). There was a significant increase in hyp compared to the medium control in all treatment groups at 24 hr, with the effect appearing to plateau at 1 ng/ml TGF-β_1 (both 1 and 10 ng/ml TGF-β_1 increased hyp production by approximately 100% compared with the medium control; $p<0.001$).

Procollagen production over the course of the experiment was calculated by subtracting the mean t_0 hyp value from all experimental hyp values (figure 3.4B). In addition, each group was corrected for changes in cell number at the end of the incubation period (figure 3.4A). The data derived from this adjustment was expressed as nmol hyp / 10^6 cells / 24 hr (see figure 3.5). Increasing the concentration of TGF-β_1 induced a dose dependent increase in procollagen production, with maximal effects observed at 1 ng/ml TGF-β_1 (2.5-fold increase compared with medium control, $p<0.001$; 10 ng/ml TGF-β_1 - 2.8-fold increase compared with medium control, $p<0.001$). The effect of increasing concentrations of TGF-β_1 on procollagen production was also assessed in the human JU77 cell line (figure 3.6). TGF-β_1 induced procollagen production in a dose-dependent manner with maximal stimulation observed at 10 ng/ml (over a 6-fold increase compared with medium control, $p<0.001$).

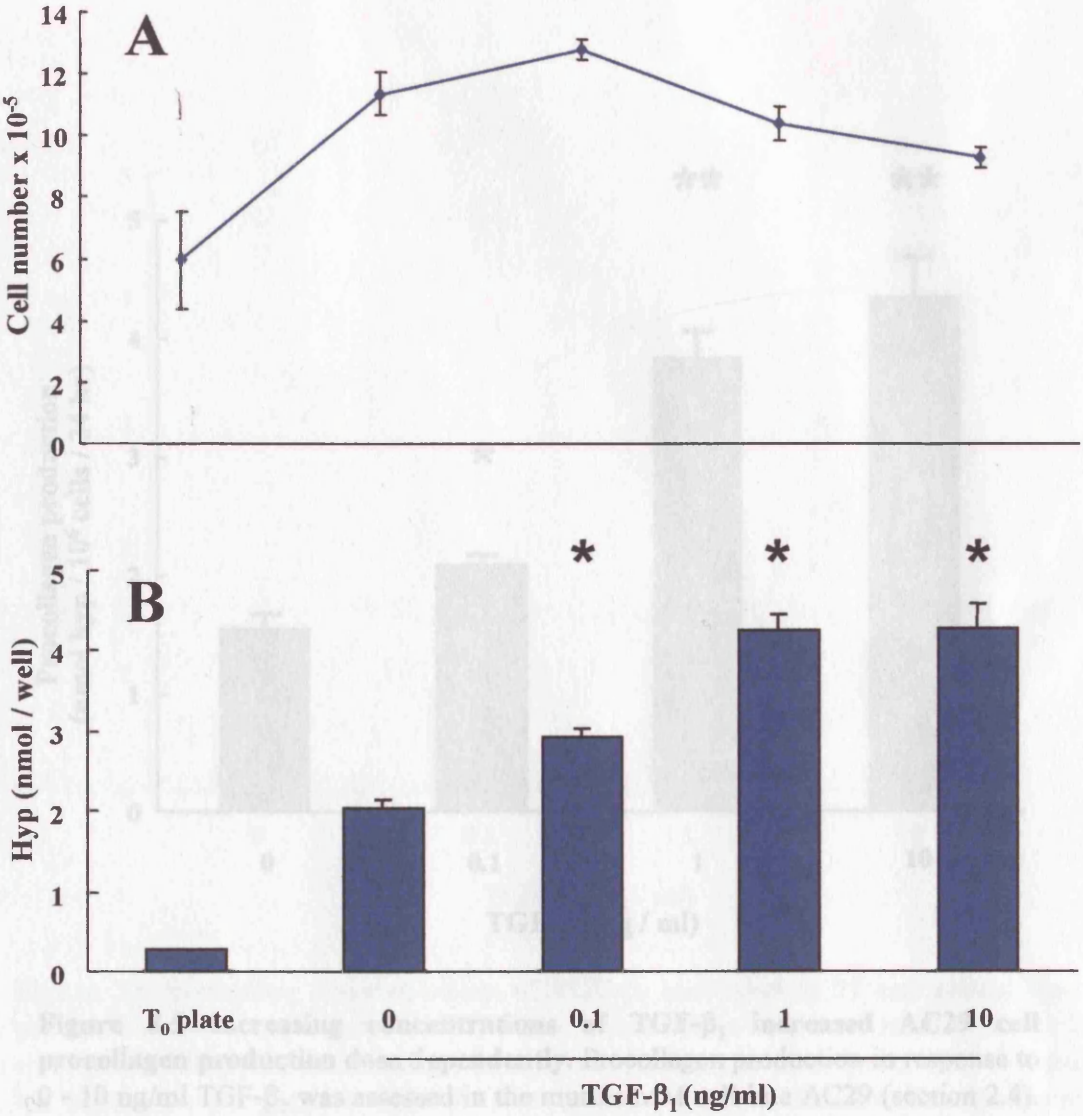


Figure 3.4 The effect of an increasing concentration of TGF-β_1 on AC29 cell number and hydroxyproline production. Confluent cultures of AC29 cells were incubated with 0 - 10 ng/ml TGF-β_1 in DMEM supplemented with 1% FBS. Cell number (**A**) and hydroxyproline production (**B**) were assessed 24 hr later. T_0 values represent the cell number and hydroxyproline at the beginning of the experiment. Statistically significant differences from medium control group are indicated by * representing $p<0.001$. Each bar represents the mean ± SEM for six replicate cultures. This data is representative of three repeat experiments.

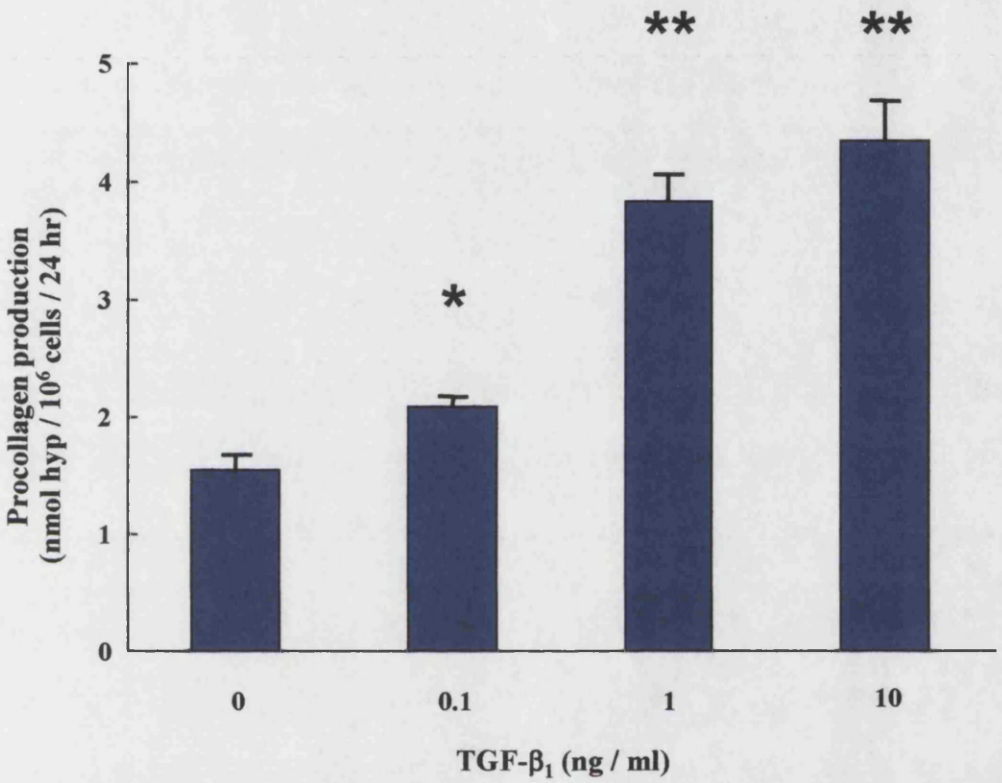


Figure 3.5 Increasing concentrations of TGF-β_1 increased AC29 cell procollagen production dose dependently. Procollagen production in response to 0 - 10 ng/ml TGF-β_1 was assessed in the murine MM cell line AC29 (section 2.4). Each bar represents the mean ± SEM for six replicate cultures. Statistically significant differences from medium control group are indicated by * and ** representing $p<0.005$ and $p<0.001$ respectively. The data shown is representative of three separate experiments.

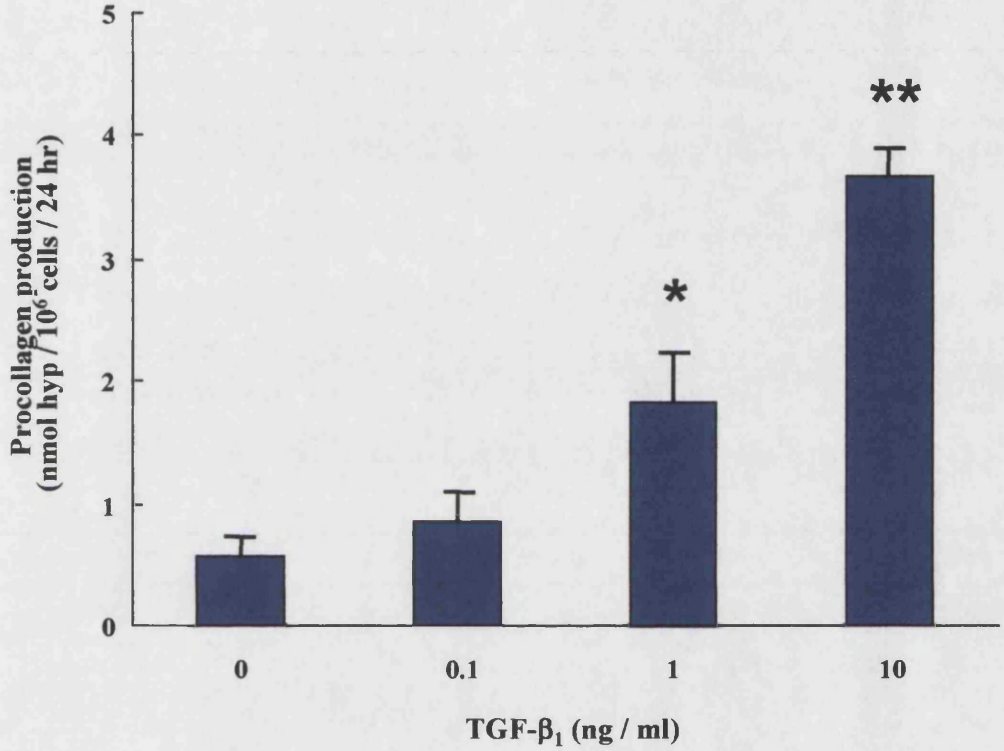


Figure 3.6 Increasing concentrations of TGF-β_1 increased JU77 cell procollagen production dose dependently. JU77 human mesothelioma cells were incubated with 0 - 10 ng/ml TGF-β_1 for 24 hr and procollagen production measured (section 2.4). Each bar represents the mean ± SEM for six replicate cultures. Statistically significant differences from medium control group are indicated by * and ** representing $p<0.05$ and $p<0.01$ respectively. This data is representative of three separate experiments.

Table 3.1 summarises the basal and TGF-β_1 induced procollagen production for murine (AB1, AB22 and AC29) and human (JU77, LO68, NO36 and ONE 58) MM cell lines, and the human normal mesothelial cell line NM20. Collagen measurements for murine CBA normal mesothelial cells were not possible due to difficulties in culturing these cells *in vitro*. However, there was over a doubling in basal procollagen production in the lowest human MM collagen procollagen producer LO68 compared with the human control mesothelial cell line NM20 (LO68 0.26±0.08, NM20 0.10±0.01 nmol hyp / 10^6 cells / 24 hr). The murine cell lines produced significantly more basal procollagen than the human cell lines, with the lowest murine procollagen producer, AB1 (1.37±0.09 nmol hyp / 10^6 cells / 24 hr) 2.5 fold higher than the greatest human procollagen producer, JU77 (0.57±0.16 nmol hyp / 10^6 cells / 24 hr, $p<0.005$).

The percent increase in procollagen production upon TGF-β_1 stimulation was also assessed. All cell lines demonstrated an increase in procollagen production upon TGF-β_1 treatment, the highest increase observed in JU77 at 10 ng/ml TGF-β_1 (approximate 540% increase compared with the medium alone control, $p<0.001$).

3.5 Discussion

3.5.1 Malignant mesothelioma cell line TGF-β production

The majority of cell lines examined produced high levels of TGF-β compared to normal CBA mesothelial cells (figure 3.3), with the murine malignant cell lines producing significantly higher levels than non-transformed murine mesothelial cells. An elevation of TGF-β production in malignant vs. non-malignant cells has also been observed by Kuwahara *et al.* (2001). Spontaneous rat mesothelioma cell lines produced 30 – 70 times more TGF-β than normal rat mesothelial cells. Interestingly, the lowest murine and human TGF-β producing cell lines, AB22 and LO68 respectively, were also the most similar morphologically to untransformed mesothelial cells (figures 3.1 and 3.2). This observation suggests that TGF-β may play a role in the process of malignant transformation as tumour cells demonstrating the highest levels of TGF-β production are morphologically more distinct from their normal counterparts. TGF-β has been demonstrated to induce transformation of fibroblasts to myofibroblasts (Desmouliere *et al.*, 1993) and keratinocytes to myofibroblasts (Jester

	Cell Line	Hydroxyproline (nmol hyp / 10^6 cells / 24 hr) according to TGF-β_1 concentration (ng/ml)			
		0	0.1	1	10
Murine	AB1	1.37 ± 0.09	1.61 ± 0.12 (17.79 ± 9.03)	2.23 ± 0.40 (62.73 ± 28.99)	2.79 ± 0.29 * (103.77 ± 21.67)
Murine	AB22	1.58 ± 0.07	3.88 ± 0.12 *** (146.13 ± 7.75)	6.29 ± 0.10 *** (298.84 ± 6.58)	8.43 ± 0.11 *** (434.54 ± 6.84)
Murine	AC29	1.55 ± 0.12	2.09 ± 0.06 ** (34.72 ± 4.10)	3.83 ± 0.23 *** (146.47 ± 15.03)	4.34 ± 0.33 *** (179.56 ± 21.44)
Human	NM20	0.10 ± 0.01	---	0.28 ± 0.01 *** (185.71 ± 5.89)	---
Human	JU77	0.57 ± 0.16	0.84 ± 0.27 (46.56 ± 47.01)	1.83 ± 0.94 * (220.32 ± 73.19)	3.66 ± 0.24 *** (540.48 ± 42.26)
Human	LO68	0.26 ± 0.08	0.37 ± 0.03 (41.16 ± 11.31)	0.55 ± 0.02 ** (106.14 ± 8.09)	0.47 ± 0.03 * (77.94 ± 13.16)
Human	NO36	0.52 ± 0.28	0.74 ± 0.09 (41.28 ±16.42)	0.87 ± 0.14 (56.51 ± 29.27)	0.83 ± 0.11 (58.89 ± 21.31)
Human	ONE58	0.34 ± 0.04	0.81 ± 0.01 *** (134.59 ± 0.82)	1.21 ± 0.09 *** (241.71 ± 28.71)	1.67 ± 0.11 *** (387.37 ± 31.21)

Table 3.1 Basal and TGF-β_1-induced procollagen production in murine MM and human normal and malignant mesothelial cell lines. Confluent cultures of cells were incubated with 0 – 10 ng/ml TGF-β_1 in DMEM supplemented with 1% FBS. Procollagen production was assessed 24 hr later. Values were corrected for cell number and procollagen production expressed as nmol hyp / 10^6 cells / 24 hr. The figures in brackets represent the percent change ± SEM compared with media control. --- indicates that measurements were unable to be made. Statistically significant differences from medium control group are indicated by *, ** and *** representing $p<0.05$, $p<0.01$ and $p<0.001$ respectively. Data is representative of at least two independent experiments.

et al., 1996), and is perhaps important in the transformation of mesothelial cells to mesothelioma.

Heterogeneity was evident in TGF-β production between the cell lines, which was consistent with findings in other studies (table 3.2). Fitzpatrick *et al.* (1994) examined TGF-β production in the same cell lines and found much higher levels of TGF-β production. However, in both studies AC29 was the highest producer of active and latent TGF-β.

Cell line	TGF-β production	This study	Fitzpatrick *et al.*, 1994	
Murine non-malignant	*Active*	0.02	-	Murine
	Total	0.36	-	
AB1	*Active*	0.13	<0.33	
	Total	4.57	<0.33	
AB22	*Active*	0.08	<0.1	
	Total	0.62	4	
AC29	*Active*	0.33	6.95	
	Total	6.46	48.38	
JU77	*Active*	0.12	2.2	Human
	Total	0.33	3.2	
LO68	*Active*	0.01	1.34	
	Total	0.04	4.08	
NO36	*Active*	0.29	5.3	
	Total	2.05	12.73	
ONE58	*Active*	0.17	5.48	
	Total	0.18	15.8	

Table 3.2 A comparison of TGF-β production by malignant mesothelioma cell lines from two independent studies. Active and total TGF-β production is expressed as ng / 10^6 cells / 24 hr.

There are several possible reasons why the two different studies do not match exactly. Media containing 1% FBS was used in the Fitzpatrick *et al.* study and conditioned

media was collected over a 48 hr period, whereas in this study serum free medium over a 24 hr period was used. The presence of growth factors such as EGF and bFGF in serum affects the proliferation of non-transfected MLEC which would interfere with the measurement of TGF-β (Abe *et al.,* 1994). These differences may have increased the amount of measurable TGF-β in the study by Fitzpatrick *et al.*. Also, different TGF-β bioassays were used to quantitate TGF-β activity, the study by Fitzpatrick and colleagues using a MLEC growth inhibition assay and this study using the more sensitive and specific transfected MLEC bioassay. Although the total values differed between the two studies, the proportion of active to total TGF-β produced by each cell line were similar in both cases.

The majority of TGF-β secreted by the cell lines was latent (figure 3.3). TGF-β is secreted in a latent form in a complex with LTBP (Taipale *et al,* 1994). In some cases LTBP acts as a chemoattractant, it has been shown to stimulate rat arterial smooth muscle cells to migrate (Kanzaki *et al.,* 1998). The ECM acts as a reservoir for various growth factors and cytokines, and is important in sequestering TGF-β (Boudreau and Bissell, 1998). Latent TGF-β is localised to the ECM by LTBP (Taipale *et al.,* 1994) creating a store of TGF-β. Proteases such as plasmin, thrombin, and elastase release matrix bound TGF-β to form a soluble pool of latent TGF-β (Taipale *et al.,* 1992, 1995). In the soluble pool, plasmin and Cathepsin D dissociate LAP and TGB-β to yield active TGF-β (Lyons *et al.,* 1988). ECM-bound TGF-β_1 can also be cleaved and released by MMPs–2, -3 or –7 (Imai *et al.,* 1997), and MMPs-2 and -9 are capable of activating pro-TGF-β_1 to localise TGF-β_1 to the cell surface (Yu and Stamenkovic, 2000). MM cells have been shown to produce these MMPs (Liu *et al.,* 2001), as well as plasminogen activator (Schoenberger *et al.,* 1987) and thrombin (Pacchiarini *et al.,* 1991). Thus, there is a large reservoir of latent TGF-β within the MM cell lines tested capable of rapidly increasing the local concentration of active TGF-β with the potential to stimulate collagen production and promote tumourigenesis *in vivo.*

3.5.2 TGF-β_1 stimulated malignant mesothelioma cells to produce procollagen in vitro

Basal and TGF-β_1-induced procollagen production was measured in a range of murine and human MM cell lines (table 3.1). TGF-β_1 is the most effective inducer of procollagen production characterised to date, and has been demonstrated to potently stimulate fibroblast procollagen mRNA production (Varga *et al.*, 1987), fibroblast procollagen protein production *in vitro* (Coker *et al.*, 1997) and the deposition of collagen *in vivo* (Roberts *et al.*, 1986). TGF-β_1 was used in this study to stimulate procollagen production in MM cell lines. All experiments were conducted in 1% serum to maintain cell adhesion and viability. Although the presence of serum may have acted as a stimulus to raise the basal level of procollagen production, a further increase in procollagen production was observed after TGF-β_1 incubation. The data obtained showed that the MM cell lines tested produced basal levels of procollagen, which was increased in a dose responsive fashion following incubation with TGF-β_1.

Of the murine cell lines, AB22 had the largest increase in procollagen production with the addition of exogenous TGF-β_1 (table 3.1; at 10 ng /ml TGF-β_1 approximate 430% increase compared to the basal level) whilst having the lowest endogenous TGF-β production (figure 3.3). Conversely, the human cell line NO36 had the lowest percentage increase in procollagen production with 10 ng / ml TGF-β_1 (table 3.1) but the highest endogenous TGF-β production (figure 3.3). This suggests that there could be an autocrine loop of active TGF-β production by MM cells stimulating endogenous procollagen production. Cell lines producing higher levels of TGF-β may have maximally stimulated endogenous procollagen production and therefore responded less to exogenous TGF-β than in those cell lines producing lower quantities of TGF-β. The role of exogenous TGF-β isoforms on MM basal procollagen production was examined later by the use of TGF-β neutralising antibodies (chapter 6).

3.5.3 Correlation between malignant mesothelioma cell morphology, TGF-β production and procollagen production

TGF-β is known to induce the transformation of fibroblasts into myofibroblasts (Desmouliere *et al.*, 1993), and is perhaps involved in the progression of normal

mesothelium to MM. The murine cell lines AB1 and AC29 have a more fibroblast-like phenotype than AB22 cells (figure 3.1), and produced relatively higher levels of TGF-β (figure 3.3) compared to the normal mesothelial control cell line. A previous study by Davis *et al.* (1992) demonstrated that AB1 & AC29 were the most tumourigenic out of a panel of murine and human MM cell lines at inducing *in vivo* tumours both s.c. and i.p., however no correlation between morphology or growth rate was found.

Individual variations in morphology and growth rates are characteristic of human and murine MM (Davis *et al.*, 1992). It has been documented that MM cell lines vary in morphology and growth (Manning *et al.*, 1991; Davis *et al.*, 1992) and in the production of various cytokines such as TGF-β (Fitzpatrick *et al.*, 1994; Kuwahra *et al.*, 2001). This heterogeneity was also observed in the MM cell lines examined in this study. The procollagen response was similar within species (human and mouse) but different between species (table 3.1). TGF-β production was consistently greater in the murine cell lines compared to the human cell lines with the exception of the human line NO36 (figure 3.3). As both groups contained cell lines of varying morphology, cell type is unlikely to be responsible for the observed differences. Interspecies variation in metabolism is likely to account for the differences seen, smaller organisms (mice) have a faster metabolism than larger animals (humans) and therefore produce proteins such as procollagen and TGF-β at a faster rate. (Vinogradov *et al.*, 2001)

3.6 Summary and conclusions

The panel of cell lines examined was heterogeneous in TGF-β and collagen production with differing morphologies. All of the cell lines produced measurable quantities of procollagen and TGF-β, with a trend towards greater levels in the murine MM cell lines. The MM cell lines also produced procollagen and TGF-β at higher levels compared with non-malignant control cells. Those cell lines producing higher concentrations of endogenous TGF-β were less responsive to TGF-β_1-induced procollagen production. There was no obvious relationship between cell morphology, TGF-β production or procollagen production, although AB22 (most morphologically similar to untransformed mesothelial cells) had the lowest TGF-β production,

suggesting a possible relationship between TGF-β production and malignant transformation.

In a panel of cell lines, including the ones characterised in this study, Davis *et al.* (1992) demonstrated that AC29 cells transplanted *in vivo* had the fastest growth rate; forming a subcutaneous 1cm diameter tumour within 25 days *in vivo*. This chapter showed that AC29 was the cell line with the highest TGF-β production and was one of the highest producers of procollagen. This suggests a possible correlation between TGF-β, procollagen production and tumour growth.

As previously stated, two key characteristics of MM are firstly that it is an extremely fibrous tumour containing a dense ECM rich in collagens, and secondly, that elevated levels of TGF-β are produced compared to normal mesothelium and other lung malignancies. The literature review in chapter one provided evidence for the independent importance of both TGF-β (section 1.3) and ECM components such as collagen (section 1.7) in promoting tumourigenesis. Also, TGF-β is the most potent inducer of collagen production characterised to date and therefore an association between TGF-β, collagen production and increased tumourigenesis could be hypothesised. Having demonstrated the increased production of procollagen in all of the MM tumour cell lines tested in this chapter the first thesis hypothesis was formulated:

The ECM is vital to the progression of malignant mesothelioma with procollagen production stimulating cell proliferation and tumour growth

Collagen production contributes to an enhanced rate of ECM deposition, which contains binding sites for LTBP and therefore leads to the formation of a reservoir of latent TGF-β. Collagen and TGF-β may be synergistic in promoting tumourigenesis, TGF-β directly stimulates collagen gene transcription whilst collagen forms part of the 'reservoir' which sequesters latent TGF-β and holds the cytokine within the local tumour microenvironment. The observation that cell lines producing a high basal

level of TGF-β had a relatively smaller increase in procollagen production in response to exogenous TGF-β suggested that an autocrine loop of TGF-β production stimulating collagen production might have been operating in the malignant cells. From these results the second thesis hypothesis was generated:

Autocrine TGF-β production by MM promotes malignant cell proliferation and tumour growth by enhancing procollagen production

The first hypothesis was tested in chapters 4 and 5, which examined the effect of inhibiting collagen production on MM cell proliferation and tumour growth in an animal model of MM. The second hypothesis was addressed by examining the effect of endogenous and exogenous TGF-β on MM cell proliferation, collagen production and *in vivo* growth, the data obtained shown in chapters 6 and 7.

Chapter Four

The role of collagen in the proliferation of malignant mesothelioma cells in vitro

4.1 Introduction

In chapter 3 it was established that MM cells produce collagen at a greater rate than normal mesothelial cells, both basally and in response to TGF-β_1. ECM components have been implicated in tumour cell growth *in vitro*. For example, MM cell lines migrate to fibronectin, laminin and collagen type IV (Klominek *et al.*, 1997) and exhibit more invasive growth through soft agar when supplemented with hyaluronan (Li and Heldin, 2001). Hyaluronan has also been observed to stimulate the migration and proliferation of MM cells (Nasreen *et al.*, 2002). Sethi and colleagues (1999) observed that ECM proteins promoted the proliferation and prevented the chemotherapy-induced apoptosis of small cell lung cancer cells. In addition, the inhibition of collagen production with a proline analogue decreased rat breast carcinoma cell proliferation (Lewko *et al.*, 1981) revealing the importance of collagen in the growth of this tumour. The role of collagen, which is a predominant ECM component, in MM growth has not been investigated. This chapter examines the importance of collagen in MM proliferation *in vitro*, and in particular aims to:

1. evaluate the effect of exogenous collagen on MM cell proliferation,
2. assess the efficacy of thiaproline as an agent to specifically inhibit collagen production,
3. and determine the effect of inhibiting endogenous collagen production on MM cell proliferation.

The MM cell line AC29 was chosen for the subsequent studies as the cells exhibited one of the highest basal levels of procollagen production, which increased approximately three fold upon stimulation with 10 ng / ml TGF-β_1 (figure 3.5). Also, studies demonstrated that AC29 cells had the fastest and most reproducible growth *in vivo* (figure 5.2), and as AC29 is a murine cell line the tumour may be easily introduced in syngeneic mice for *in vivo* experiments.

4.2 Effect of collagen matrices on mesothelioma cell proliferation

Following the characterisation of the basal and induced levels of MM cell line procollagen, by hyp amino acid production in chapter 3, the effect of growing AC29 cells on a collagen matrix on cell proliferation was assessed *in vitro* using ^{3}H-TdR

incorporation studies. Figure 4.1 demonstrates the effect of an increasing concentration of fibrillar rat tail collagen type I substrate on AC29 DNA synthesis. Thymidine incorporation was dose-dependently increased to a maximum of 11230 ± 520 d.p.m. / well at 25 ng / well of collagen type I compared to 7090 ± 340 d.p.m. / well for medium alone control. This represented an approximate 60% increase compared with medium control, $p<0.001$. At higher collagen type I concentrations there was a significant increase in thymidine incorporation above the medium control, but values were lower compared with the 25 ng / well group (250 ng / well; approximate 40% increase compared to medium control, $p<0.001$ and also a significant decrease compared with 25 ng / well group, $p<0.05$; 500 ng / well group having similar values).

The effect of increasing concentrations of murine basement membrane collagen type IV substrate on AC29 cell proliferation was also investigated (figure 4.2). Only 25 ng / well of collagen type IV induced a small but significant increase in thymidine incorporation (approximate 20% increase compared with control, $p<0.05$).

4.3 Determination of the cytotoxic effects of thiaproline in vitro

Having determined that MM cells produce procollagen basally and that exogenous collagen can influence AC29 cell proliferation, the effect of inhibiting endogenous procollagen production, using the proline analogue thiaproline, was investigated. Firstly, the cytotoxic effect of thiaproline on AC29 cells was assessed in order to find a range of non-toxic doses for *in vitro* use. Figure 4.3 demonstrates the effect of an increasing concentration of thiaproline on subconfluent AC29 cell viability (as used in cell proliferation assays). The mean A_{540} at t_0 was 0.15 ± 0.01, representing the number of cells at the beginning of the assay. With an increasing concentration of thiaproline a dose dependent decrease in A_{540} compared to the medium control was observed. Concentrations of thiaproline greater than 10 mM exhibited a lower A_{540} than the t_0 value, demonstrating that there were fewer cells than at the beginning of the experiment (20 mM; approximately 15% cell death, $p<0.05$; 30 mM - approximately 85% cell death, $p<0.001$; 40 mM - approximately 98% cell death, $p<0.001$). Concentrations above 40 mM caused total cell death.

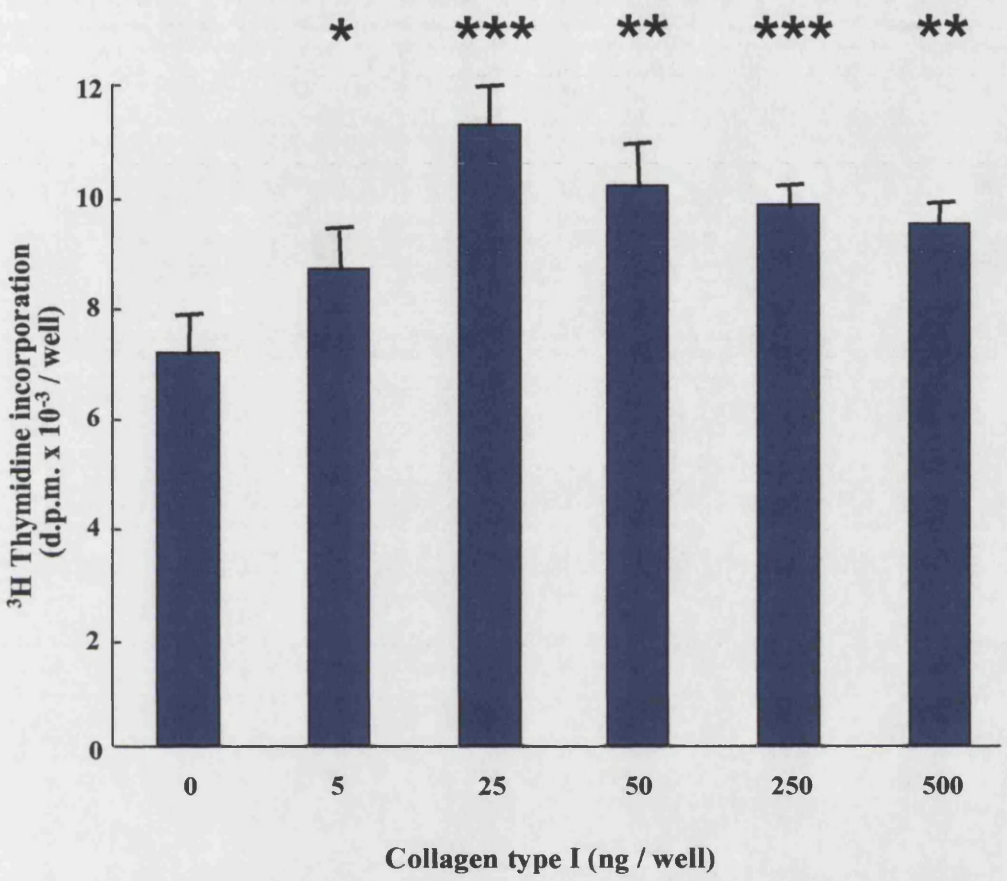


Figure 4.1 Increasing concentrations of collagen type I substrate increased AC29 DNA synthesis. AC29 DNA synthesis was assessed 24 hr after being cultured on collagen type I matrices at a range of concentrations between 0 and 500 ng / well (section 2.2.2). Each bar represents the mean ± SEM for six replicate cultures. Statistically significant differences from 0 ng collagen / well control group are indicated by *, ** and *** representing $p<0.05$, $p<0.005$ and $p<0.001$ respectively. This data is representative of 2 experiments.

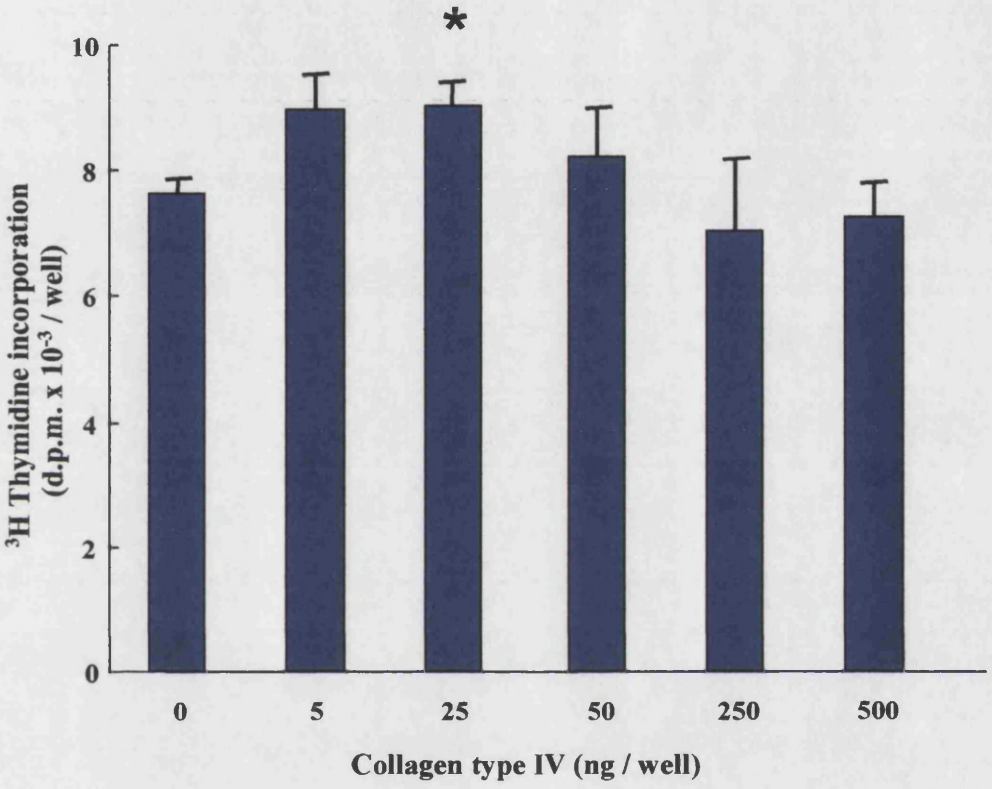


Figure 4.2 Collagen type IV substrate had minimal effects on AC29 DNA synthesis. AC29 DNA synthesis was assessed 24 hr after being cultured on 0 - 500 ng / well collagen type IV matrices (section 2.2.2). Each bar represents the mean ± SEM for six replicate cultures. This data is representative of 2 experiments.

* $p<0.05$ compared with 0 ng collagen / well control group.

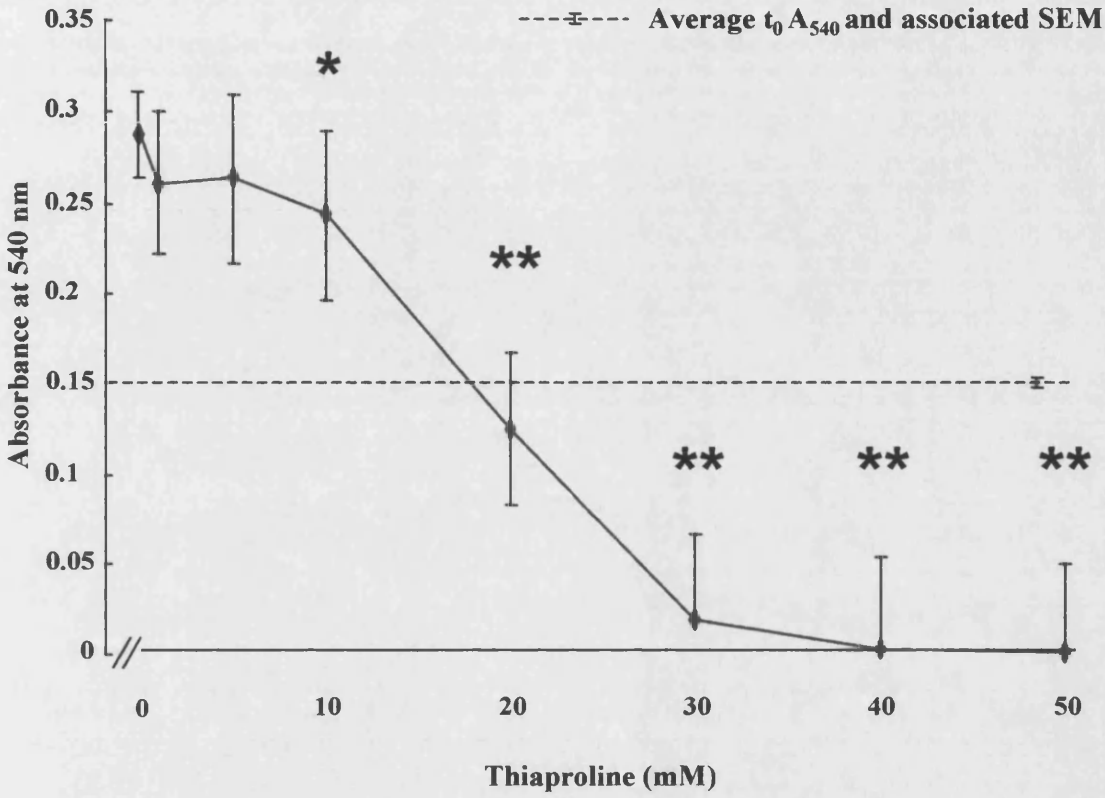


Figure 4.3 Assessment of subconfluent AC29 cell viability following thiaproline treatment. Cell viability was assessed spectrophotometrically by uptake of neutral red and measuring light absorbance at 540 nm 24 hr after treatment with thiaproline at a range of concentrations between 0 and 50 mM. Each point represents the mean ± SEM of six replicate cultures. The broken line represents the mean absorbance value at t_0 ± SEM. Statistically significant differences from medium control group are indicated by * and ** representing $p<0.05$ and $p<0.001$ respectively. Data representative of three separate experiments.

The effect of thiaproline on confluent AC29 cell viability (as used in procollagen production assays) was also assessed, as shown in figure 4.4. The mean t_0 A_{540} was significantly greater than that of the subconfluent assay (approximately 230% higher, $p<0.001$) indicating a greater cell number. Concentrations of thiaproline between 1 – 30 mM did not significantly reduce A_{540} compared with the medium control. Concentrations greater than 30 mM significantly reduced A_{540} compared with both the medium control (40 mM; approximate 50% reduction, $p<0.001$) and the mean t_0 value (similar values as compared with the medium control). This data indicated that thiaproline was toxic above 30 mM on confluent cells.

The effect of thiaproline on AC29 cell viability was assessed using an alternative cytotoxicity assay measuring LDH release. Figure 4.5A shows that an increasing concentration of thiaproline on subconfluent cells caused a dose dependent decrease in intact cell layer LDH (representing live cell number) and a concentration dependent increase in supernatant LDH (representing lysed cells). When expressed as a percentage of released LDH over total LDH (figure 4.5B), there was a significant increase in LDH release to 7.8% at 5 mM ($p<0.01$) and to 11.5% at 10 mM ($p<0.001$) compared to medium control (6.3%). Although this was suggestive of cytotoxic effects at lower concentrations of thiaproline than previously observed, the percentage increases obtained in figure 4.5B were minor.

Figure 4.6A demonstrates the cytotoxic effect of thiaproline on confluent AC29 cells. Thiaproline induced a dose dependent increase in supernatant LDH release, although did not cause a significant decrease in cell layer LDH until over 10 mM. Figure 4.6B demonstrates that at 10 mM and above, thiaproline caused a significant increase in the percentage of total LDH released (an increase from the medium control value of 12.8% to 16.1%, $p<0.001$). Based on these data from the two different cytotoxicity assays, subsequent experiments were performed using thiaproline at concentrations between 0 and 10 mM.

4.4 Effect of thiaproline on AC29 cell procollagen and non-collagen protein production

Basal and TGF-β_1-induced procollagen production in response to a range of non-toxic

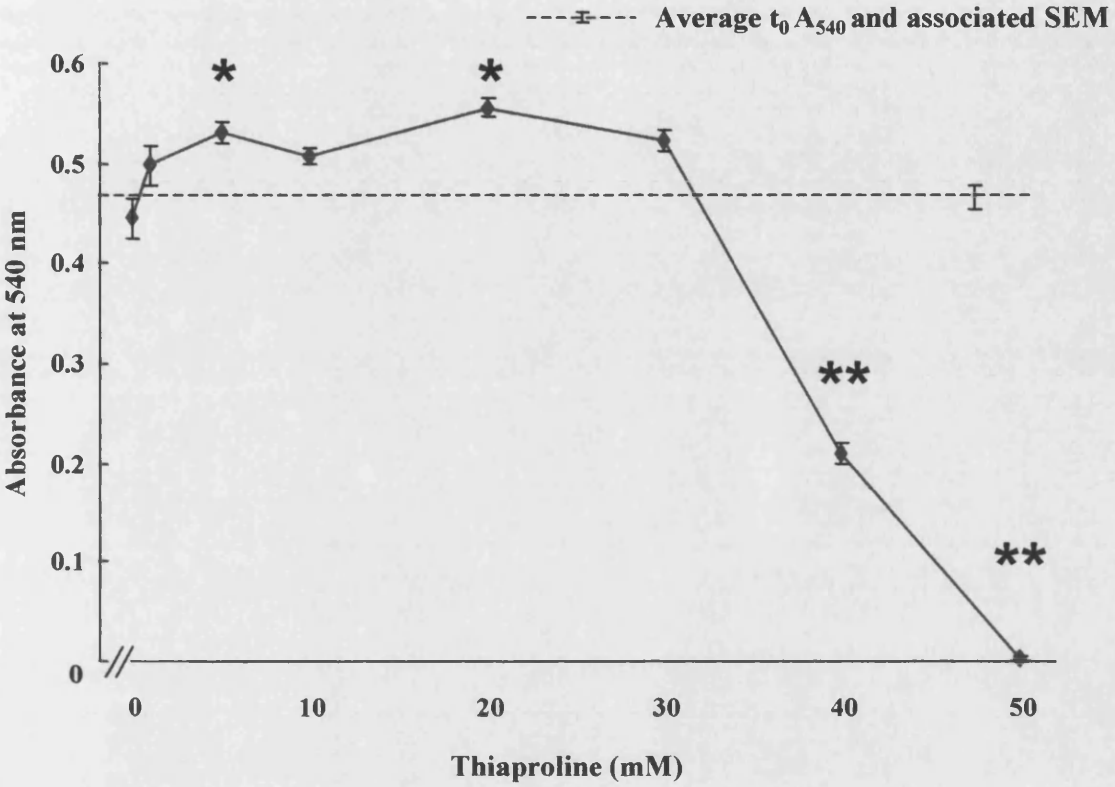


Figure 4.4 The effect of increasing concentrations of thiaproline on confluent AC29 cell viability. Cell viability was assessed spectrophotometrically 24 hr after treatment with thiaproline at a range of concentrations between 0 and 50 mM (section 2.3.1). Each point represents the mean ± SEM of six replicate cultures. The broken line represents the mean absorbance value at t_0 ± SEM. Statistically significant differences from medium control group are indicated by * and ** representing $p<0.05$ and $p<0.001$ respectively.

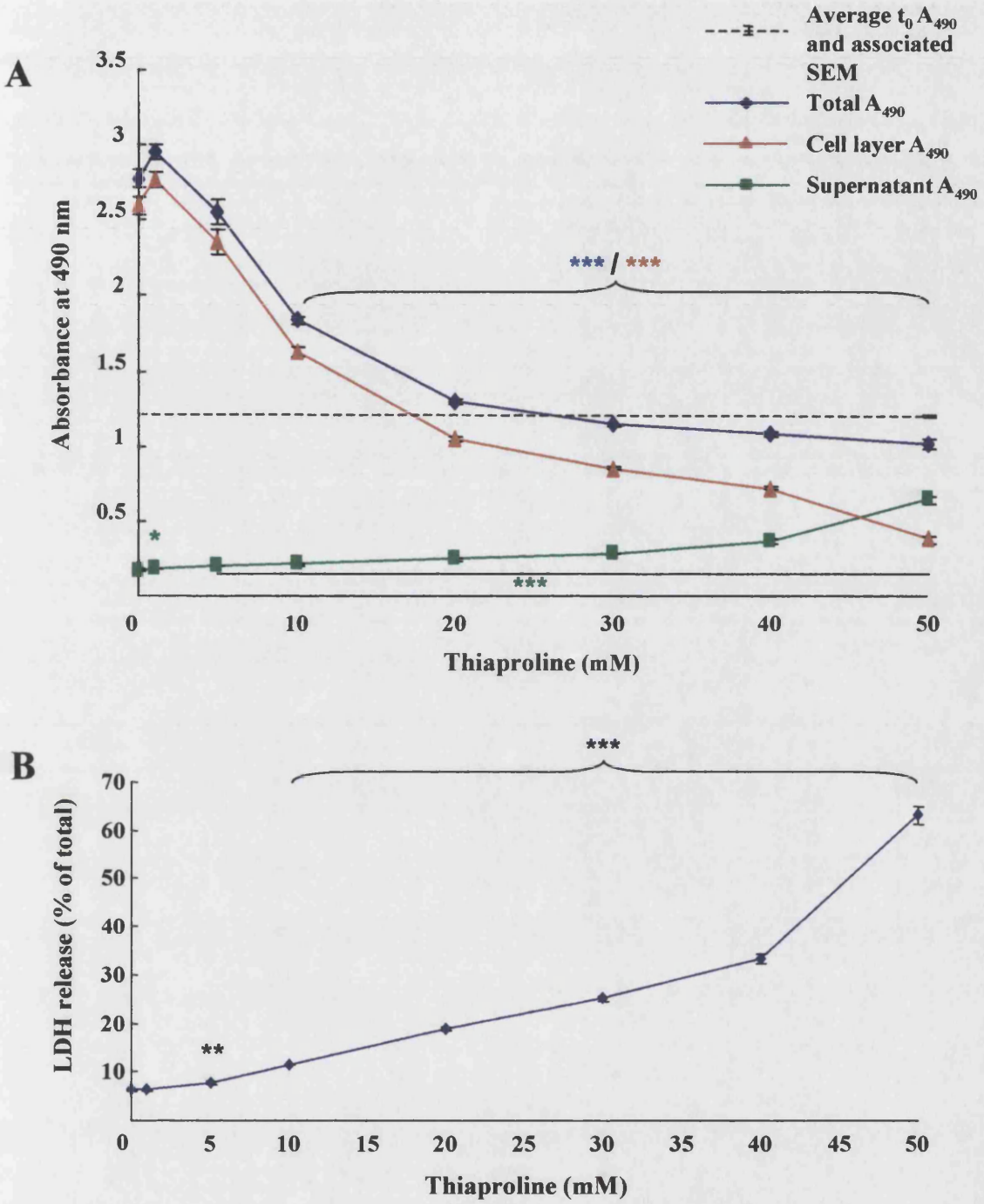


Figure 4.5 Effect of thiaproline on subconfluent AC29 lactate dehydrogenase (LDH) release. **A** demonstrates thiaproline (0 - 50 mM) cytotoxicity towards cultured AC29 cells after 24 hr, measured by release of cytoplasmic lactate dehydrogenase in the supernatant, in the lysed cell monolayer and as a combined total. **B** represents percentage of total LDH release. Values represent means ± SEM of six replicate cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. medium control. Data representative of three separate experiments.

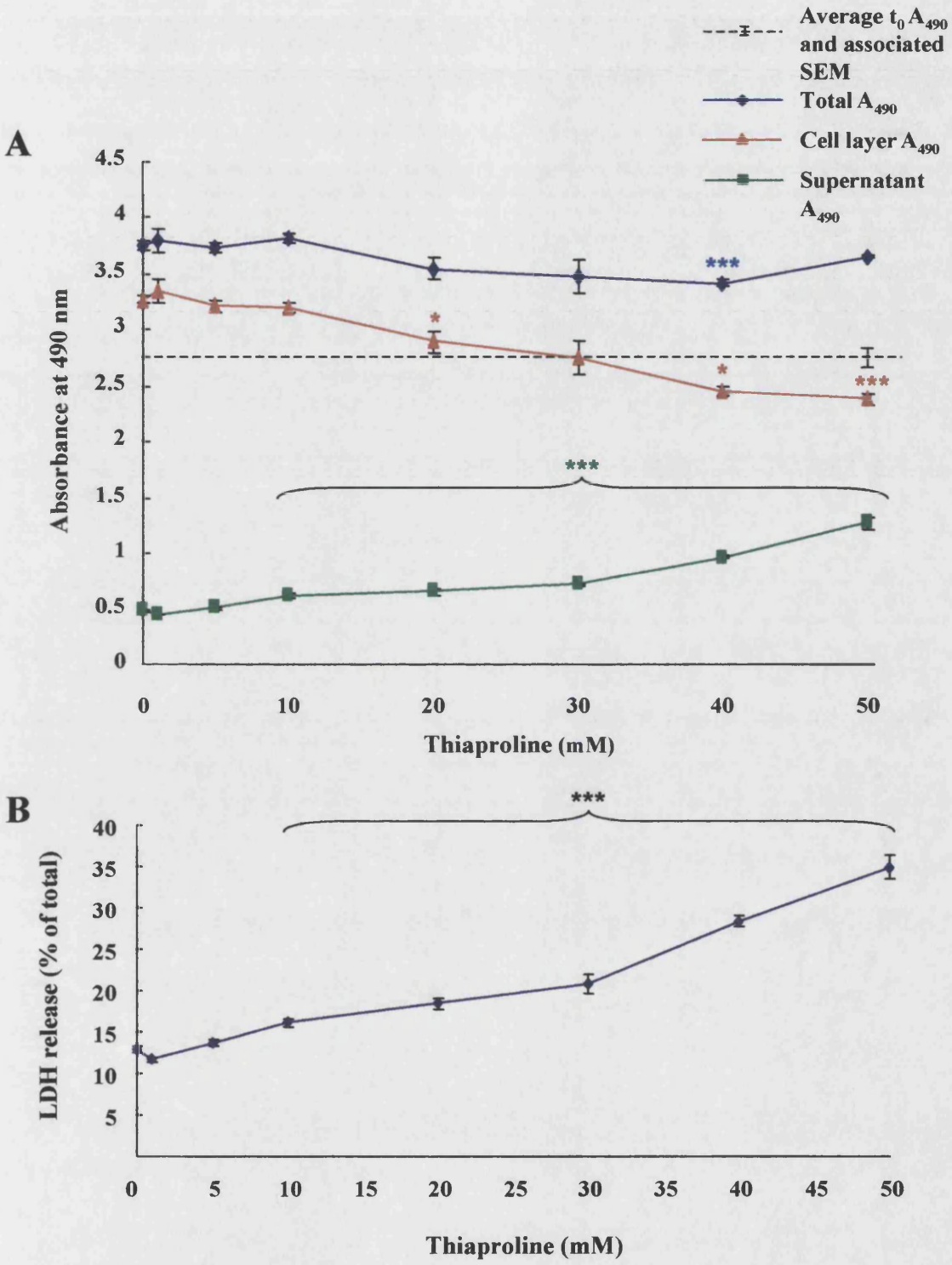


Figure 4.6 Effect of thiaproline on confluent AC29 LDH release. The cytotoxic effects of thiaproline (0 - 50 mM) on AC29 cells was measured by release of supernatant and lysed cell monolayer cytoplasmic lactate dehydrogenase after 24 hr in culture (**A**). **B** represents percentage of total LDH release. Values represent means ± SEM of six replicate cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. medium control. Data representative of two independent experiments.

doses of thiaproline was measured in AC29 cells (figure 4.7). TGF-β_1 stimulated procollagen production by approximately 150% compared to medium control (3.83±0.23 compared with 1.55±0.12 nmol hyp / 10^6 cells / 24 hr respectively, $p<0.001$). With increasing concentrations of thiaproline both basal and TGF-β_1-induced procollagen production were decreased in a dose-dependent manner with a maximal effect at 10 mM (compared to media control basal reduction of approximately 50%, $p<0.001$; TGF-β_1-treated reduced by approximately 65%, $p<0.001$). At each concentration of thiaproline there was also a significant increase in procollagen production over the basal level with TGF-β_1 treatment.

To assess the specificity of thiaproline, a comparison of the effects of thiaproline on procollagen production and non-collagen protein production in AC29 cells was made (figure 4.8). At both 1 and 10 mM, thiaproline decreased non-collagen protein production by approximately 15%. However, neither of these reductions were significant compared with the medium control. Thiaproline at 1 mM inhibited both procollagen and non-collagen protein production to a similar extent (approximately 20% and 15% respectively). However, at 10 mM thiaproline there was a much greater inhibition of procollagen (approximately 50%, $p<0.001$ compared with medium) than non-collagen protein production (approximately 15%). There was a significant difference between the inhibition of procollagen and non-collagen protein production at 10 mM ($p<0.001$) suggesting that at this concentration thiaproline has a greater specificity towards inhibition of procollagen protein production.

4.5 Effect of thiaproline on cell proliferation in vitro

Having assessed the effect of non-toxic doses of thiaproline on AC29 cell procollagen and non-collagen protein synthesis, the effect of thiaproline on cell proliferation was examined. The results in figure 4.9 demonstrate that ^{3}H-thymidine incorporation is reduced in a dose-dependent manner with an increasing concentration of thiaproline. Concentrations of 1, 5 and 10 mM thiaproline resulted in decreases of approximately 20, 35 and 75% respectively ($p<0.005$, $p<0.001$ and $p<0.001$ respectively, compared to medium control).

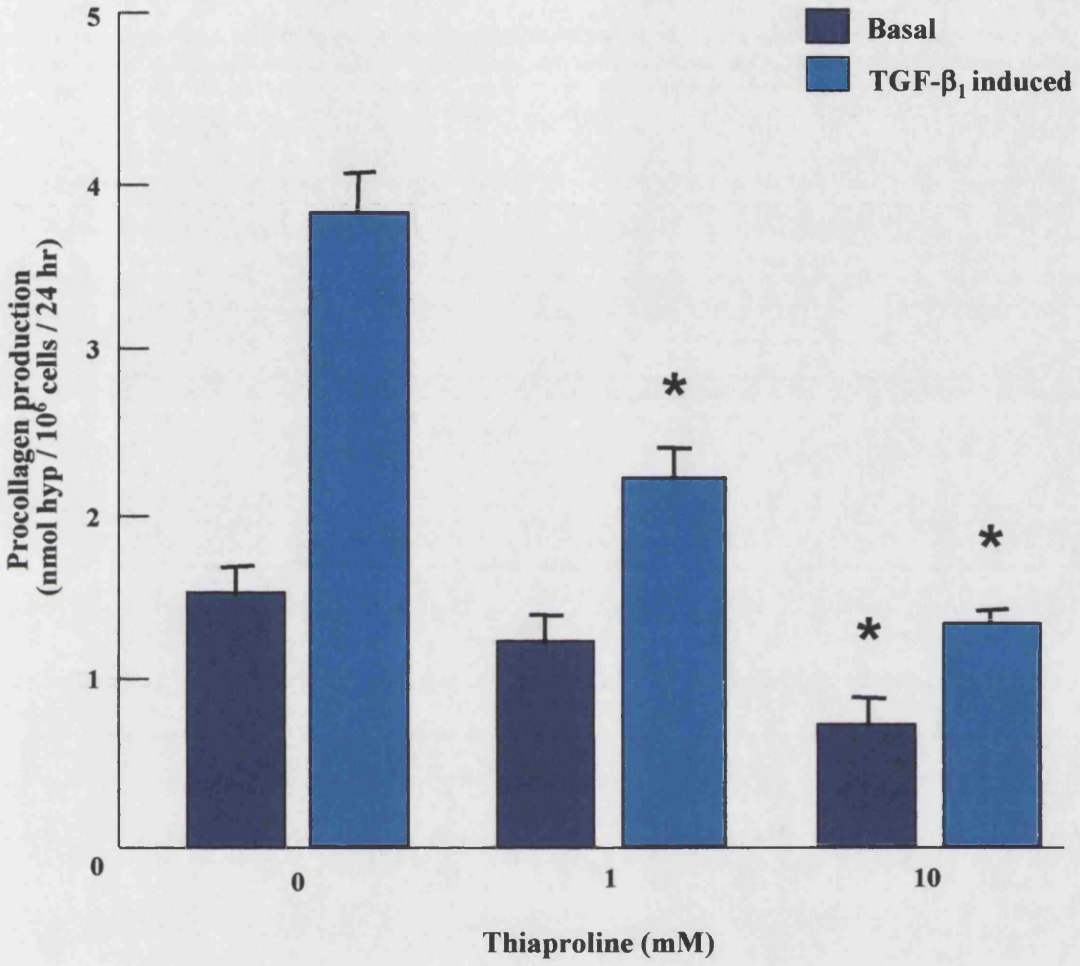


Figure 4.7 Increasing concentrations of thiaproline decreased basal and TGF-β_1-induced AC29 cell procollagen production in a dose-dependent manner. Confluent cultures of AC29 cells were incubated with thiaproline at a range of concentrations between 0 and 10 mM with and without 1 ng/ml TGF-β_1. After 24 hr procollagen production was assessed (section 2.4). Each bar represents the mean ± SEM for six replicate cultures. * denotes a significant difference of $p<0.001$ compared with the relevant media control group (0 mM thiaproline). Data is representative of at least four independent experiments.

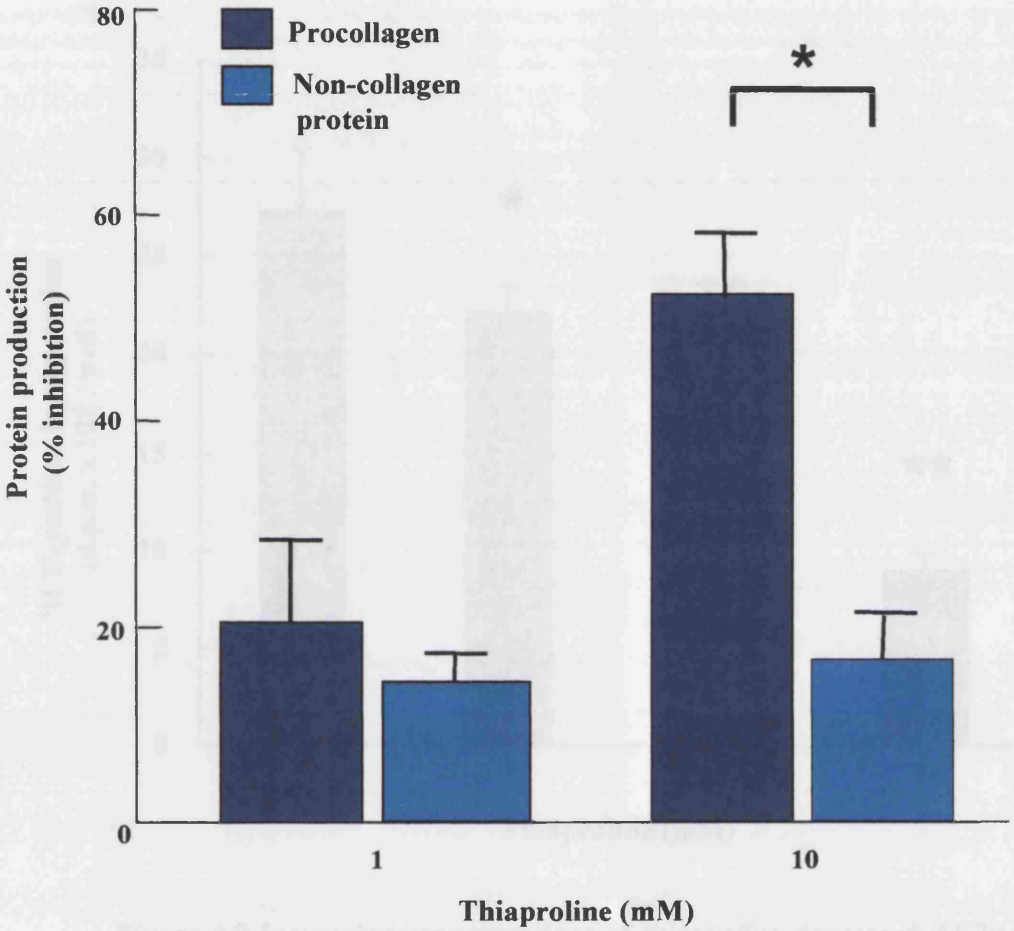


Figure 4.8 A comparison of the effect of increasing concentrations of thiaproline on AC29 cell procollagen and non-collagen protein production. Confluent AC29 cells were incubated with thiaproline at 0, 1, and 10 mM. Procollagen and non-collagen protein production were assessed 24 hr later. The level of protein production was expressed as percentage inhibition compared with the media control (taken as 0% inhibition). Each bar represents the mean ± SEM for six replicate cultures. * denotes a significant difference of $p<0.001$. Data representative of two separate experiments.

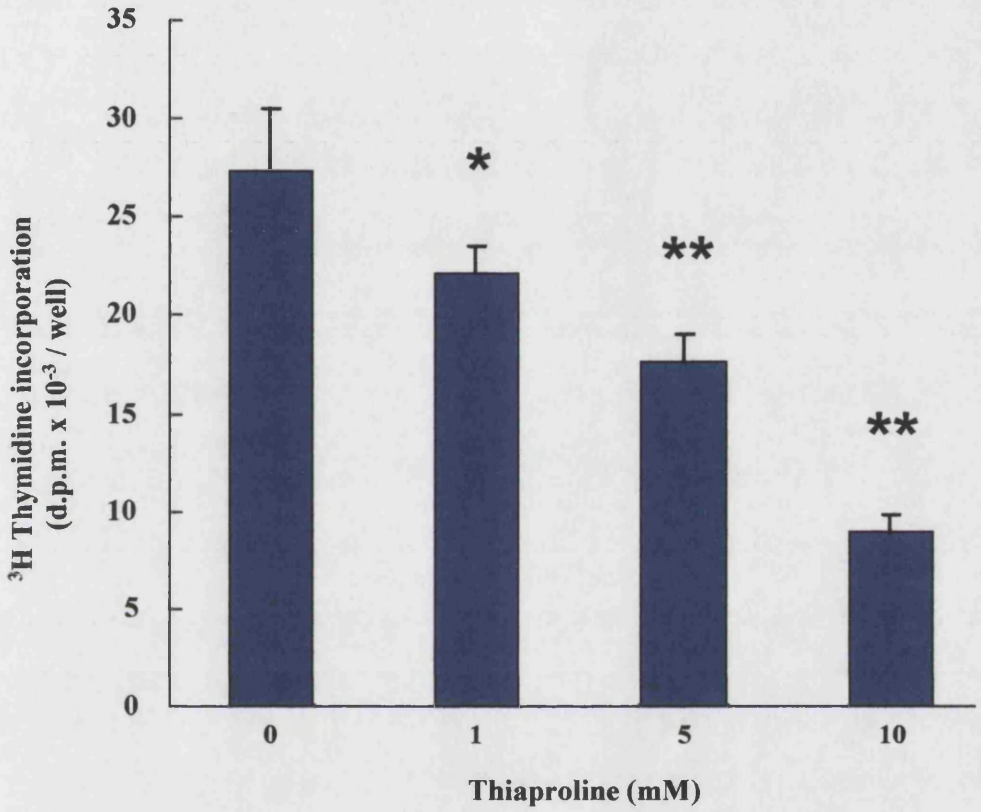


Figure 4.9 Increasing concentrations of thiaproline decreased AC29 DNA synthesis in a dose-dependent manner. AC29 DNA synthesis was assessed 24 hr after culture with 0 - 10 mM thiaproline (section 2.2.1). Each bar represents the mean ± SEM for six replicate cultures. Statistically significant differences from medium control group are indicated by * and ** representing $p<0.005$ and $p<0.001$ respectively. Data representative of two separate experiments.

4.6 Discussion

4.6.1 Exogenous collagen type I increased the in vitro rate of AC29 cell proliferation

The effect of collagen on MM cell proliferation was examined by culturing cells on collagen type I and type IV matrices. Figure 4.1 demonstrates that cells grown on collagen type I had an increased rate of proliferation at all concentrations examined, compared with medium control. Although the MM cells were murine in origin and the collagen type I was extracted from rat this is unlikely to affect the experiment as there is a high degree of homology between rat and mouse collagen α1(I) genes. Additionally, Miller and Gay (1982) reported that interspecies differences between a given type of collagen chain are minimal. Murine collagen type IV caused a relatively small increase in cell proliferation compared with the medium control (figure 4.2).

The difference in the proliferative capacity of collagen type I and type IV could be due to the differing nature of these two collagens. Type I is a fibrillar interstitial collagen commonly found in tumour matrices and may be important in tumour growth. Kauppila *et al.* (1998) have established histologically that there was an increase in type I and type III procollagen mRNA in malignant human breast cancer compared to benign tumours. They also observed that the expression of both type I and type III procollagens increased with an increasing grade of tumour malignancy, suggesting that an increase in fibrillar collagen is associated with worse tumourigenicity.

Ylisirnio *et al.* (2001) have shown that elevated serum levels of the carboxyterminal telopeptide of type I collagen (ICTP) and Tissue Inhibitor of Metalloproteinse (TIMP) 1 are associated with poor prognosis in various lung cancers. This suggests that an increased rate of collagen type I production in these cancers may contribute to malignancy and lead to a poor prognosis. Further evidence to support a role for collagen type I in tumour growth was reported by Simojoki *et al.* (2001). They demonstrated that increased serum levels of the ICTP are associated with a worse patient prognosis in ovarian cancer.

Collagen type IV is associated with the basement membrane and therefore may not be as important for MM cell proliferation as interstitial collagens. A study by Han *et al.* (1997) demonstrated that a cell binding domain of collagen type IV inhibited the *in*

vitro proliferation of human melanoma cells. Applying this observation to MM, it is possible that a normal basement membrane prevents cell proliferation, but upon malignant transformation an increase in interstitial collagens (seen in malignant breast carcinomas by Kauppila *et al.*, 1998) may stimulate cell growth, as demonstrated in figure 4.1.

An *in vitro* study of the effect of various ECM substrates on human MM invasiveness through a three-dimensional gel demonstrated that growth on collagen type I substrate led to an increased rate of invasiveness when compared with collagen type IV (Ehlers *et al.*, 2002). This study suggests that varying substrates have different effects on the invasive potential of MM cells, and supports the observations in figure 4.1 and 4.2.

4.6.2 Inhibition of malignant mesothelioma cell procollagen production reduced the rate of cell proliferation in vitro

To determine the effect of endogenous procollagen production on the proliferation of MM cells *in vitro*, cells were exposed to increasing concentrations of the proline analogue thiaproline. Lubec *et al.* (1994, 1997) demonstrated a reduction in collagen accumulation in the heart and the kidney basement membrane of db/db spontaneous diabetic mice fed thiaproline. Thiaproline is incorporated into protein in competition with proline and prevents chain elongation resulting in truncated peptides which are rapidly degraded (Hortin and Boime, 1983). As non-collagen protein contains approximately 5% proline and collagen contains 15 – 20% proline there should be a preferential inhibition of collagen production.

The use of an amino acid analogue carries the risk of non-specific inhibition, as the analogue can be incorporated into any protein containing the amino acid. An alternative approach to specifically inhibit collagen production would be the use of an inhibitor to a step unique in the processing of collagen (see review by Kagan, 2000). Targeting enzymes specific to collagen production (lysyl oxidase and prolyl 4-hydroxyalse) have been attempted, but finding compounds capable of effective collagen inhibition that are non-toxic has proven difficult. The inhibitor β-aminopropionitrile (BAPN) inactivates lysyl oxidase and prevents collagen fibrillogenesis (Tang *et al.*, 1983). However the compound has been reported to be

toxic, and cross-linkage formation in elastin is also catalysed by lysyl oxidase and therefore would be affected by BAPN. Franklin *et al.* (2001) have described the use of a novel series of phenanthrolinones, compounds capable of inhibiting prolyl 4-hydroxylase. However, these inhibitors are toxic at effective concentrations *in vivo*, and this is a reoccurring problem with such compounds. In this study, thiaproline was chosen as an inhibitor of collagen production, as in other studies appropriate concentrations of this analogue have been shown to be effective both *in vitro* and *in vivo* without toxicity. Thiaproline was initially assessed to determine a suitable range of concentrations for *in vitro* use.

Thiaproline has previously been shown to be non-toxic *in vitro* at a dose of 120 μM (Gosalvez *et al.*, 1979). Figure 4.3 demonstrates that thiaproline up to a concentration of 5 mM did not affect cell viability compared with the medium control. Thiaproline at a concentration of 10 mM marginally decreased the number of viable cells compared with the medium control, but this number of cells was much greater than the t_0 value. The decrease in cell number with 10 mM thiaproline may have been due to an inhibition of cell proliferation as opposed to cytotoxic effects. Concentrations of thiaproline greater than 10 mM reduced the number of viable cells below the t_0 value, indicative of cell death.

Thiaproline had no significant effect on confluent cell cultures compared with the medium control up to 30 mM (figure 4.4). Above 30 mM thiaproline caused a decrease below the t_0 value suggesting cytotoxicity. These data demonstrate that AC29 cells have a better tolerance for thiaproline at confluence compared with subconfluence. This may be explained by the fact that at confluence the cells are more tightly packed together, surrounded by more ECM and have a lower metabolism than subconfluent cells.

These data were verified using an alternative assessment of cytotoxicity measuring LDH release. Figure 4.5A demonstrates that increasing concentrations of thiaproline on subconfluent AC29 cells increased the number of lysed cells, but decreased the total number of cells. Due to the decrease in total cell number, when supernatant LDH was expressed as a percentage of the total possible LDH release (figure 4.5B) there

were significant increases compared with the medium control at concentrations of thiaproline above 1 mM, anomalous with the previous observation in figure 4.3.

These data could be interpreted to show that thiaproline is toxic on subconfluent cells at concentrations of 1 mM and above. However, morphologically, thiaproline treated cells were still adherent and appeared unchanged from the medium control up to concentrations of 10 mM. Increasing concentrations of thiaproline also increased LDH release when applied to confluent AC29 cell cultures (figure 4.6B), although again the morphology of the cells remained unchanged at 10 mM.

Kubo *et al.* (1999) assessed the cytotoxicity of eosinophil granule major basic protein on tumour cell lines using five different methods and found strikingly discrepant results amongst the assays. The discordant results between the two cytotoxicity assays performed in the current study may have been due to the different characteristics of cell viability that each measured. The neutral red assay evaluated lysosomal membrane integrity in terms of retention of the neutral red dye whereas the LDH release assay assessed the intactness of the cell membrane. These data taken together (figures 4.3 – 4.6) demonstrated that thiaproline began to have slight toxic effects at 10 mM.

Thiaproline is an agent that inhibits protein elongation (Hortin and Boime, 1983) and at high concentrations it would be expected to lead to cell damage due to insufficient protein production. At a concentration of 10 mM thiaproline showed approximately 30% toxicity compared with the medium control (figure 4.6B) and inhibited non-collagen production by approximately 20% (figure 4.8). However, at the same concentration of 10 mM, thiaproline inhibited procollagen production by approximately 50% (figure 4.8). These data demonstrated that thiaproline although having slight toxic effects still exhibited specificity towards procollagen inhibition. Therefore, the highest concentration of thiaproline used for *in vitro* experimentation was 10 mM.

Hyp accounts for approximately 12% of the primary amino acid sequence of procollagen (Laurent *et al.*, 1981). This amino acid is not at a significant level in other proteins with the exception of those indicated in table 4.1. MM cells in culture have

not been shown to produce these proteins and, as their localisation indicates, they would be unlikely to be present in MM cell cultures. Also, being the predominant constituent of the ECM, collagen is likely to contribute to the majority of measurable

Protein	Hyp content (%)	Cell / tissue localisation	Major Function
Acetylcholinesterase	5 (Mays and Rosenberry, 1981)	Synaptic / neuromuscular junctions (Schumacher *et al.*, 1986)	Hydrolyses the neurotransmitter acetylcholine to terminate synaptic signalling
Complement protein C1q	4 (Reid, 1979)	Macrophages and monocytes (Ramadori *et al.*, 1986; Martin and Loos, 1988)	Key molecule in the recognition of immune complexes
Elastin	0.9 – 1.9 (Paz *et al.*, 1982)	Blood vessels, skin, lung, elastic cartilage (reviewed by Kadar *et al.*, 2002)	Provides elasticity to tissues
Surfactant apolipoprotein A	5.9 (Hawgood, 1989)	Lung alveolar surface (Hawgood and Clements, 1990)	Allows efficient gas exchange to occur, aids immune functions of lung

Table 4.1 The localisation and function of significant hyp containing proteins other than collagen

hyp. Therefore, measuring hyp content from cell cultures is an accurate reflection of procollagen production. Incubation of cells with thiaproline resulted in a dose-dependent significant decrease in basal and TGF-β_1-induced AC29 procollagen production (figure 4.7), demonstrating the ability of thiaproline to inhibit both basal and TGF-β_1 induced procollagen production.

To establish that the effect of thiaproline was specific to hyp containing proteins, of which collagen is the major contributor, cells were incubated with thiaproline and procollagen production was compared with non-collagen production (figure 4.8). At a 1 mM concentration of thiaproline there was a similar inhibition of both procollagen and non-collagen protein production. Incubation with 10 mM thiaproline led to a significant increase in the level of procollagen inhibition (approximately a two and a half fold increase) whereas the level of non-collagen protein inhibition remained unchanged compared with that at 1 mM. This data suggests that at a higher concentration of thiaproline there was a selective significant increase in the inhibition of procollagen production compared with non-collagen protein production, that remained unchanged.

Having established that AC29 cells produced procollagen, that cell proliferation was stimulated on an exogenous collagen substrate, and that non-toxic doses of thiaproline reduced AC29 procollagen production, the effect of thiaproline on AC29 cell proliferation was examined. Figure 4.9 demonstrates that thiaproline significantly inhibited AC29 cell proliferation *in vitro*, and as there was selective inhibition towards procollagen production at the higher end of the range of concentrations of thiaproline, the decrease in proliferation was consistent with inhibition of collagen production.

4.7 Summary and conclusions

Exogenous collagen type I substrate enhanced the proliferation of AC29 cells, as did collagen type IV, although to a lesser extent. Thiaproline had minimal toxic effects at a concentration of 10 mM on subconfluent AC29 cells and no toxic effects on confluent cells. At non-toxic doses thiaproline inhibited AC29 procollagen production whilst having a negligible effect on non-collagen protein synthesis. These

observations confirm the ability of thiaproline to specifically inhibit collagen production. Incubation with thiaproline resulted in a dose-dependent decrease in AC29 cell proliferation. These data suggest an important role for collagen in AC29 cell proliferation *in vitro*, and led to the investigation of the role of collagen in the growth of AC29 solid tumours *in vivo*, described in the following chapter.

Chapter Five

The role of collagen in malignant mesothelioma tumour growth in vivo

5.1 Introduction

As demonstrated in chapters 3 and 4, MM cells produce collagen and are stimulated to proliferate when grown on collagen substrate. Increasing evidence implicates a role for ECM in tumour growth. Lewko *et al.* (1981) demonstrated that inhibiting collagen production with a proline analogue reduced the growth of rat mammary tumours. Sethi and colleagues (1999) observed that ECM proteins promoted the proliferation and prevented the apoptosis of small cell lung cancer cells. These effects were inhibited by the use of antibodies preventing cell-ECM interactions. Whalen and Ingber (1989) prevented cell implantation and tumour formation in surgical wounds by inhibition of tumour cell attachment to ECM with RGD containing peptides. The studies cited above suggest an important role for cell-ECM interactions in tumour growth and highlight the potential benefit of inhibiting ECM production in attenuating tumour spread. The aim of this following chapter is to test the hypothesis that the ECM, in particular collagen, is vital to the growth and progression of MM. Specifically, this chapter aims to:

1. find a suitable *in vivo* model of MM for collagen studies,
2. and to assess the effect of inhibiting collagen production, a major ECM component, on MM tumour growth.

5.2 Effect of thiaproline on murine tumour growth models of malignant mesothelioma

Chapter 4 demonstrated that non-toxic doses of thiaproline reduced AC29 cell procollagen production and cell proliferation *in vitro*. Thiaproline was further examined in this chapter by examining its effects on *in vivo* MM tumour growth. Figure 5.1 demonstrates the effect of thiaproline on AC29 intraperitoneal tumour growth. Female CBA mice were inoculated intraperitoneally with 10^6 syngeneic AC29 cells and thiaproline was given orally (as previously described by Lubec *et al.*, 1994) in drinking water at a dose of approximately 100 mg/kg/day. Controls consisted of an AC29-injected group which received untreated water, as did a further control group with no MM cell inoculation. Tumours were observed as numerous scattered nodules, predominantly attached to the mesentery, and some to the surface of the abdominal organs. When culled, the entire abdominal contents were removed.

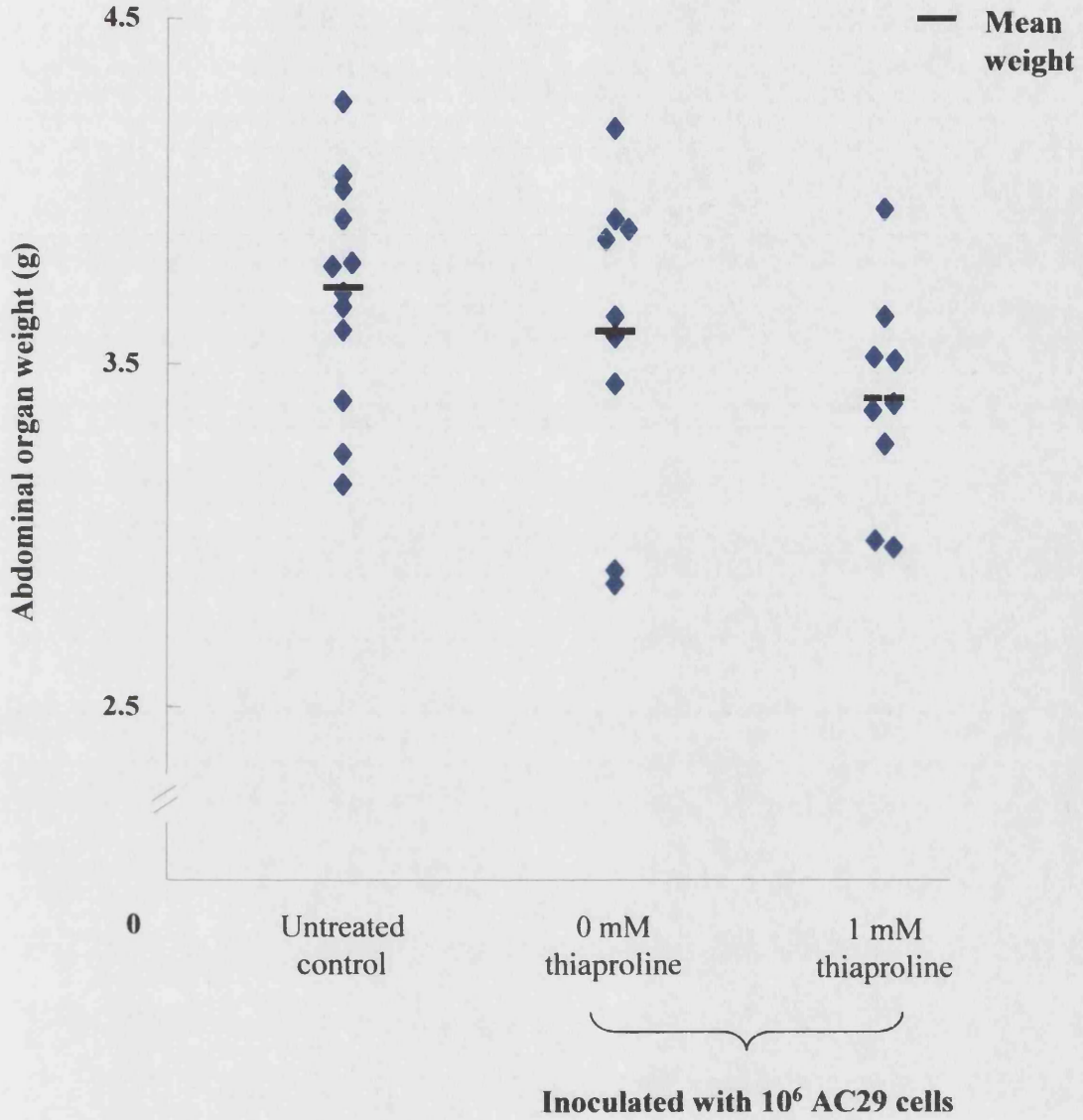


Figure 5.1 Intraperitoneal model of MM. Female CBA mice were injected intraperitoneally with 10^6 AC29 cells in 100µl of DMEM, the untreated control receiving DMEM alone. The animals were sacrificed after 18 days and the entire abdominal contents surgically removed and weighed. Each point represents the abdominal content weight for an individual animal.

At 18 days the untreated control had a mean abdominal organ weight of 3.71 ± 0.10 g, the untreated injected group 3.59 ± 0.15 g and the treated group 3.40 ± 0.10 g. Although there was a trend towards decreased weight in the treated group, the large spread of abdominal organ weights in each group and high ratio of abdominal content weight to tumour weight made this model unsuitable to study the effect of thiaproline on MM *in vivo* growth.

The *in vivo* growth rates of the murine cell lines were further assessed in a subcutaneous murine model. Female BALB/C mice were inoculated subcutaneously on both hind flanks with 10^6 syngeneic AB1 or AB22 cells and CBA mice were injected with syngeneic AC29 cells. When the tumours reached an external diameter of 1 cm, the animals were culled (several of the tumours reached this size at 18 days, the remainder at 25 days). The tumour growth pattern was characterised by a lag phase of 5 – 7 days, during which time no tumours were detected, followed by a rapid growth to macroscopic tumours. The two longest perpendicular dimensions of the excised tumour, measured by microcallipers, were used to calculate tumour area. Figure 5.2 demonstrates that AC29 had the fastest rate of growth between 18 to 25 days. In addition to this experiment, previous studies by Davis *et al.* (1992) showed that out of a panel of murine and human MM cell lines AB1 and AC29 were the most tumourigenic at inducing subcutaneous and intraperitoneal *in vivo* tumours. Also, the same study determined that AC29 had the fastest *in vivo* growth rate. Therefore subsequent experiments in this thesis used AC29 cells to induce tumours as an *in vivo* model of MM.

However, the tumours obtained during the pilot experiment (figure 5.2) were very nodular (figure 5.3) making calculations of tumour volume based on the measurement of length and breadth inaccurate. The possibility of using tumour weight as a measurement of growth was examined (figure 5.4). Using weight provided a more accurate means of measuring tumour size allowing the measurement of tumours from an earlier time-point. Small tumours that were difficult to measure through the skin could be surgically removed and weighed on a microbalance. Consequently, all subsequent experiments used AC29 cells to induce tumours and tumour weight was used as a measure of tumour size.

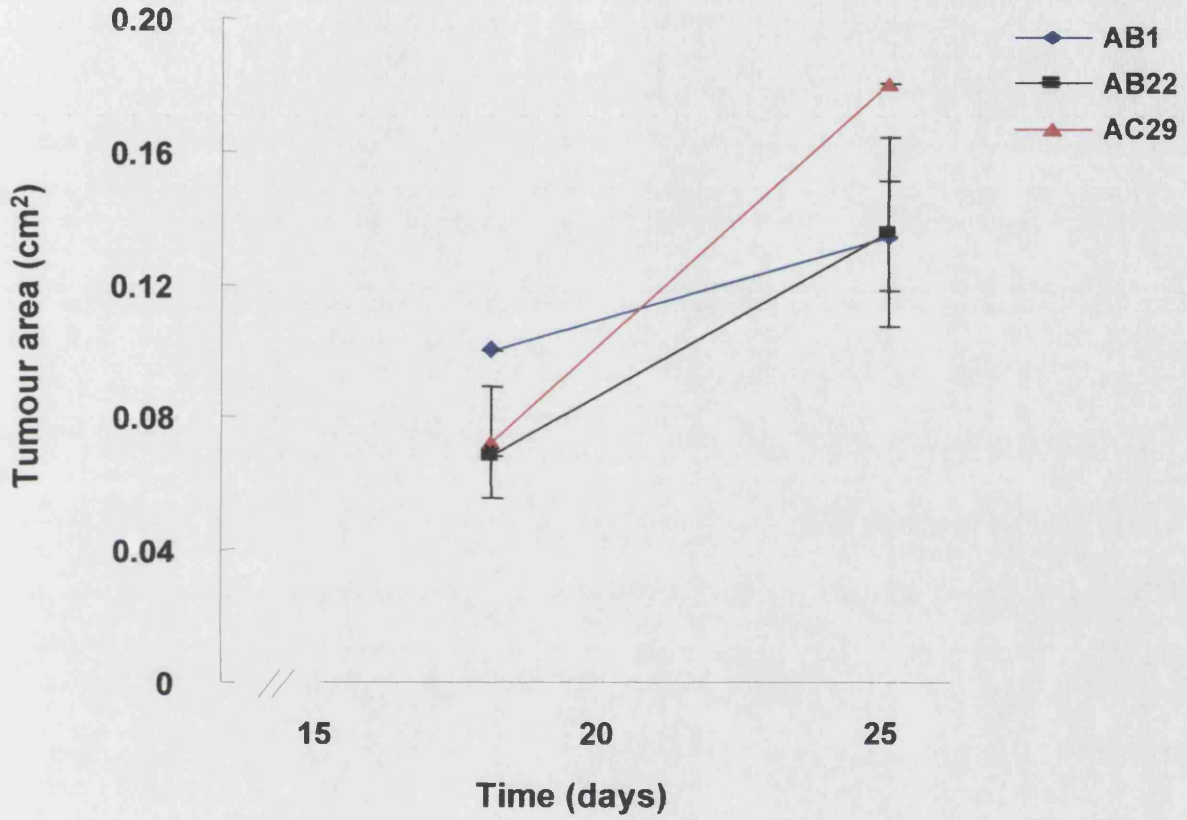


Figure 5.2 Pilot study to assess *in vivo* growth of murine MM cell lines. Female CBA mice were inoculated with 10^6 AB1, AB22 and AC29 cells on both hind flanks. The induced tumours were grown to an external diameter of 1 cm (measured by microcalipers) and then surgically removed. The excised tumours were measured in the two longest dimensions and their area calculated. The data is presented as tumour area against time of excision. Each point represents the mean of at least two tumours.

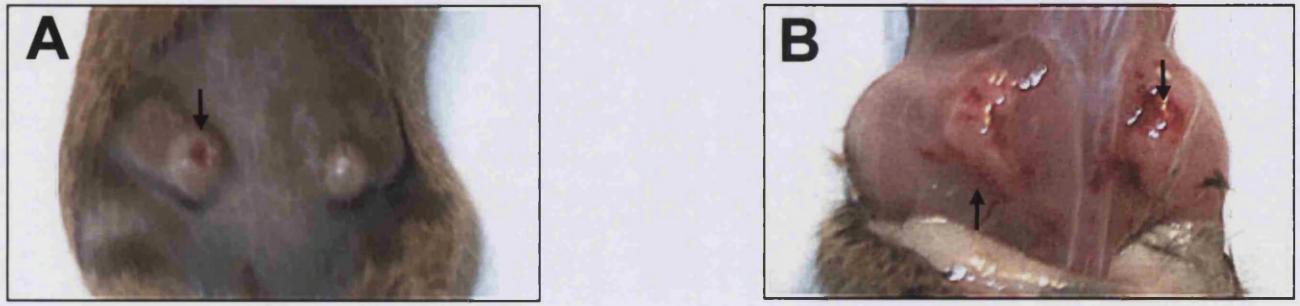


Figure 5.3 Subcutaneous model of MM. Female CBA mice were injected subcutaneously with 10^6 AC29 cells in 100µl of DMEM. **A** The animals were sacrificed when tumours were approximately 1 cm in diameter and started to ulcerate through the skin (indicated by arrow). **B** An incision was made and the skin peeled back revealing the underlying tumours. The tumours appeared highly vascularised, blood vessels indicated by arrows.

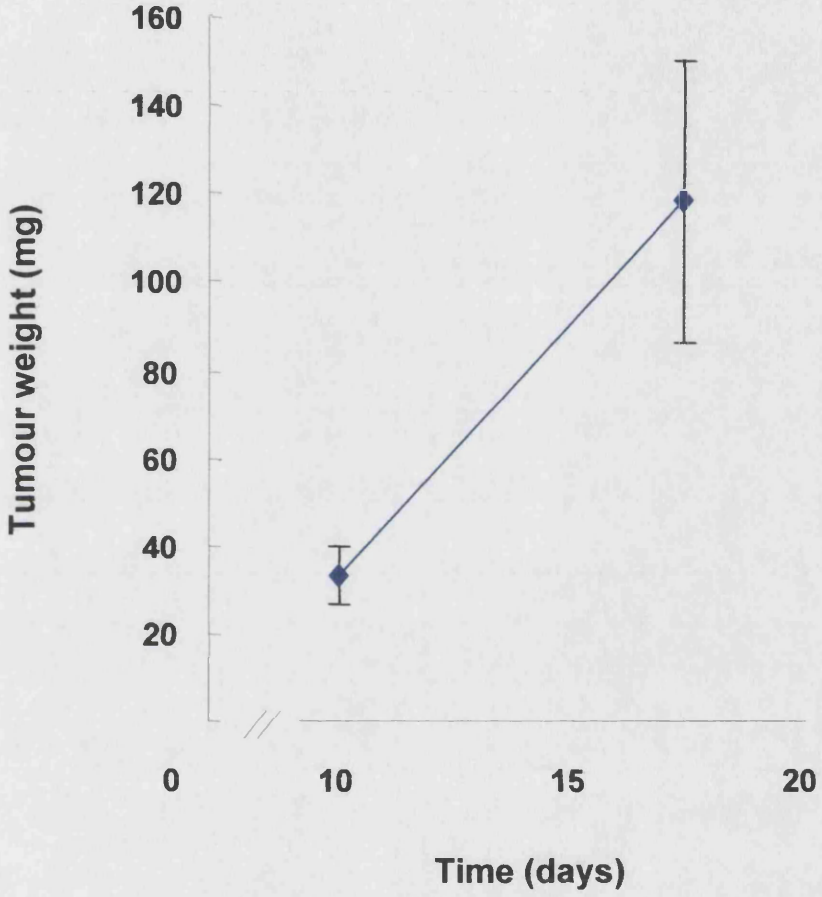


Figure 5.4 Subcutaneous model of MM using weight as a measurement of tumour growth . Female CBA mice were injected intraperitoneally with 10^6 AC29 cells in 100µl of DMEM. Groups of animals were sacrificed at 10 and 18 days, the tumours surgically removed and weighed. Each point represents the mean weight of 10 tumours .

Subcutaneously inoculated animals were given thiaproline orally in drinking water at a dose of approximately 100 mg/kg/day. The control group received untreated water. The tumours were excised at 10 and 18 days. Although most tumours were located subcutaneously, a few had invaded either the skin or muscle. When excising tumour tissue, care was taken to extract only tumour masses. The tumours were generally a single large mass, although occasionally several tumour masses were observed. All of the tumours exhibited a high degree of vascularity with prominent blood vessels supporting the tumours (figure 5.3B).

Figure 5.5 shows the effect of thiaproline on tumour weight at 10 days. The thiaproline treated group had a median tumour weight of 10.5 mg (range 5 - 12 mg). The median tumour weight of the control group was 58 mg (range 30 – 105 mg). This represented a reduction in median tumour weight of over 80% with thiaproline treatment, $p<0.001$. The 18 day weights are displayed in figure 5.6. The median tumour weight of the treated animals was 106 mg (range 46 – 187 mg), the control group also had a median tumour weight of 106 mg (range 27 - 326 mg).

In order to confirm that thiaproline was not affecting animal weight and water consumption, these variables were measured at regular intervals throughout the course of the experiment in the control and thiaproline treated animals. Figure 5.7A demonstrates the weight of thiaproline treated and control animals over 17 days. The mean animal weight increased by approximately 10% for the treated group and by approximately 12% in the control group over the course of the experiment. These values are in accordance with the supplier's growth curves for the weight of these animals. There was no significant difference in animal weight between the two groups at any time point. Figure 5.7B shows the effect of thiaproline treatment on water consumption in treated and control animals over 17 days. Water consumption between treatment groups was similar over time.

To be confident that the tumours were exposed to thiaproline, the circulating levels of thiaproline in the blood plasma of control and treated animals were assessed. Figure 5.8 shows a standard curve of thiaproline in water measured by HPLC (example chromatogram shown in figure 5.9A), and demonstrates the ability to detect thiaproline from a concentration of 0.05 mM. A correlation coefficient of 0.997

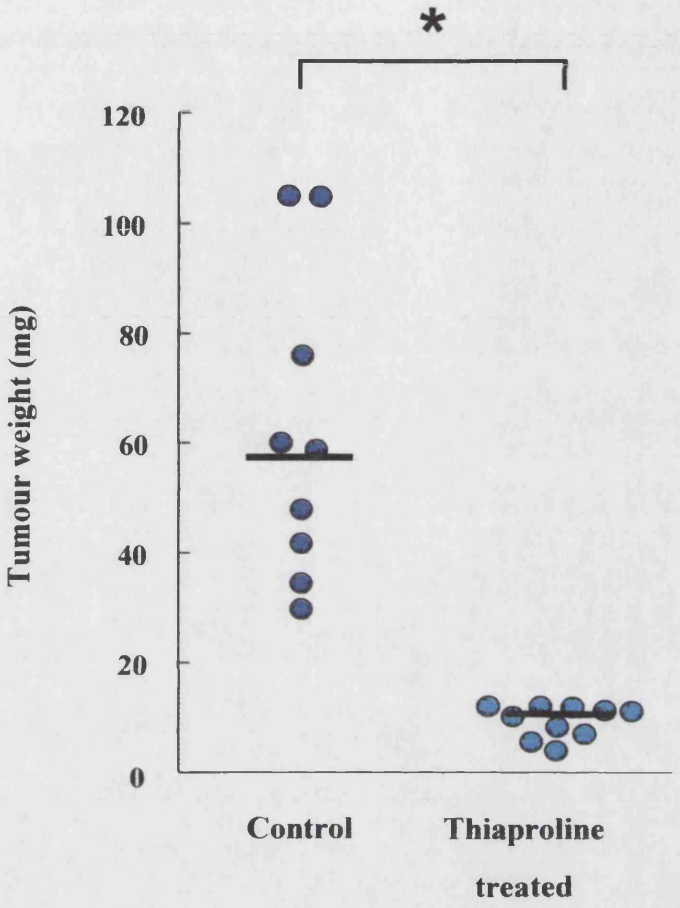


Figure 5.5 Thiaproline reduced subcutaneous mesothelioma tumour growth at 10 days. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells on both hind flanks. The control group had free access to drinking water, the treated group received water supplemented with thiaproline. The induced tumours were excised at 10 days and weighed. Each point represents an individual tumour. The median tumour weight is indicated by the horizontal bar. * denotes a significant difference of $p<0.001$.

The data are representative of a further two independent experiments, expressed as median weight (range) in mg:

Control 20 (9 - 76); thiaproline treated 9 (2 - 41); $p<0.01$

Control 80 (9 - 365); thiaproline treated 31 (6 - 116); $p<0.05$

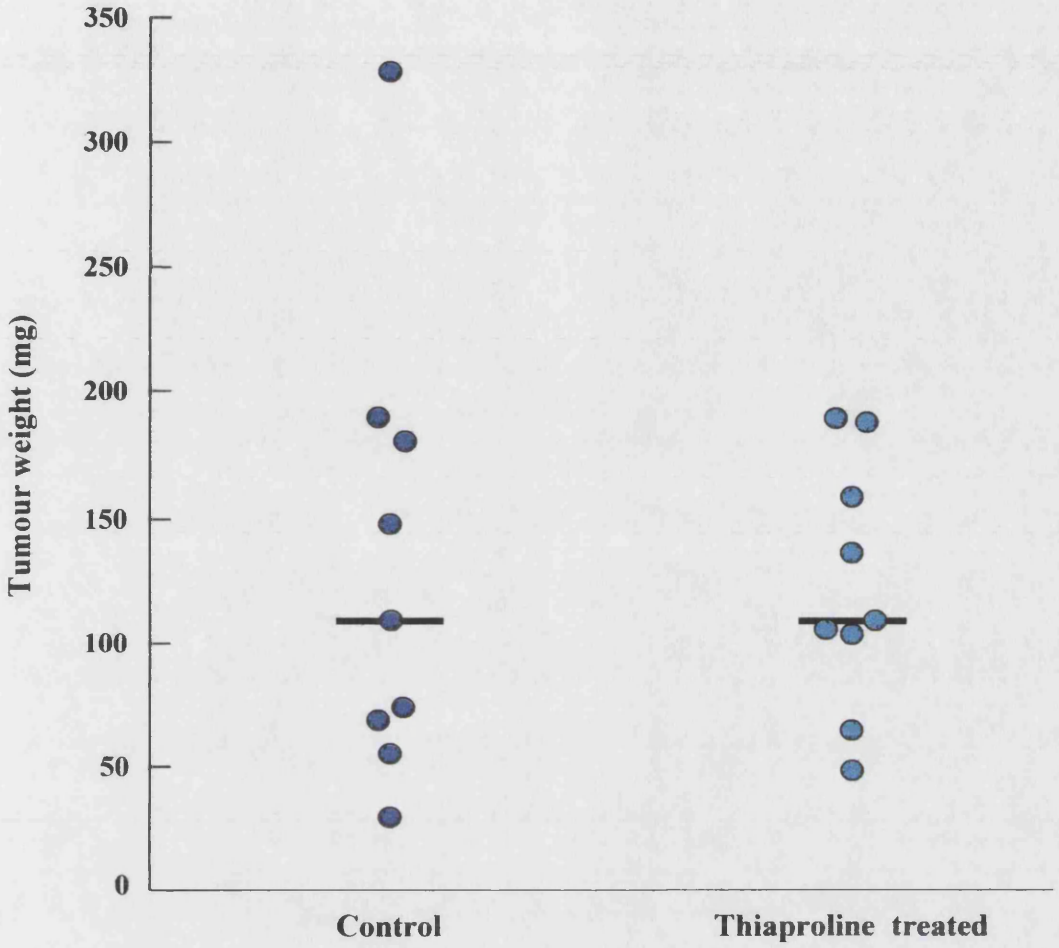


Figure 5.6 Thiaproline had no effect on subcutaneous mesothelioma tumour growth at 18 days. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells on both hind flanks. The control group had free access to drinking water, the treated group received water supplemented with thiaproline. The induced tumours were excised at 18 days and weighed. Each point represents an individual tumour. The median tumour weight is indicated by the horizontal bar.

These results are representative of a further independent repeat experiment, expressed as median weight (range) in mg:

Control 50 (1 - 365); thiaproline treated 31 (5 - 116)

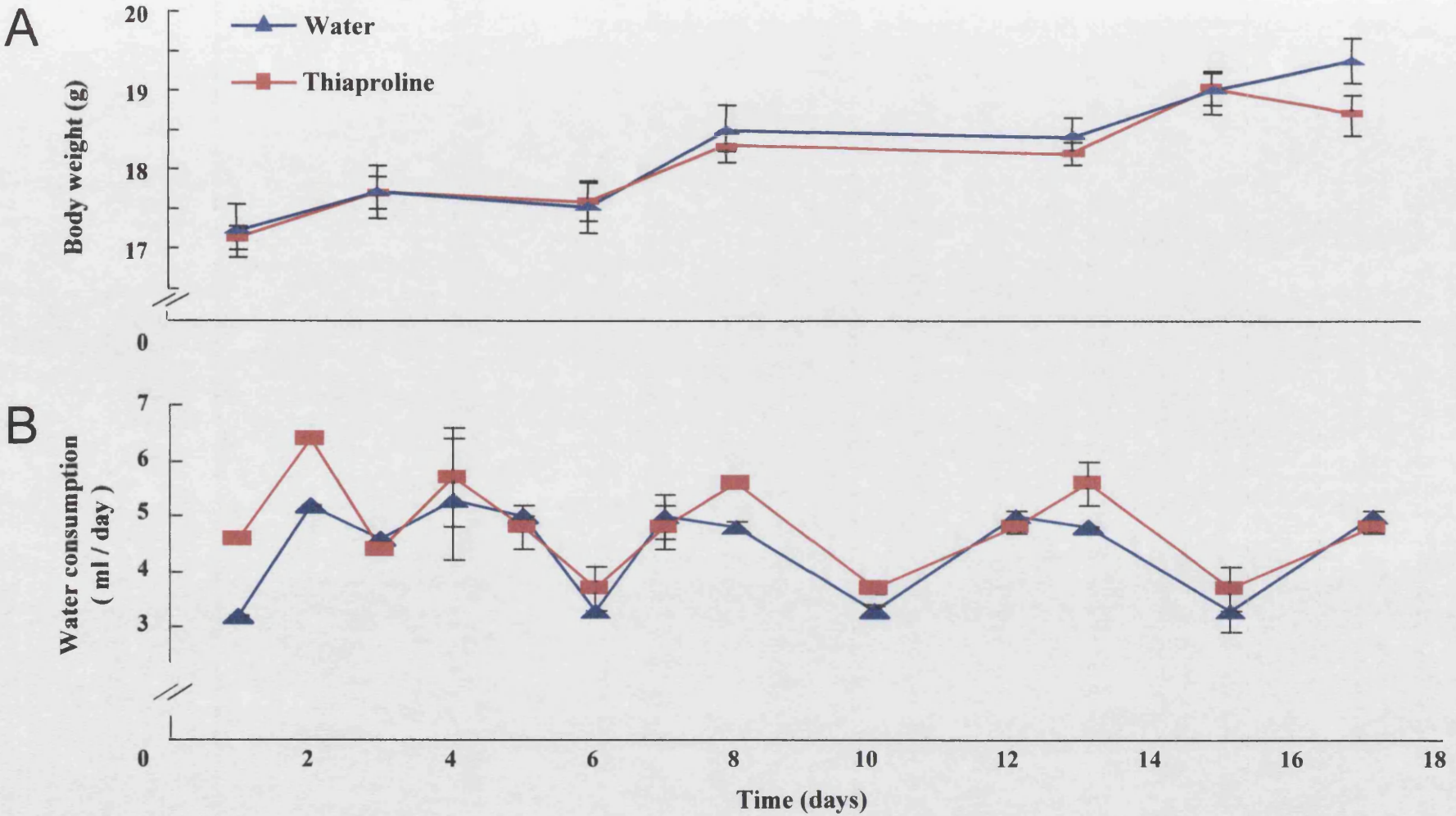


Figure 5.7 Thiaproline had no effect on animal weight or water consumption. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells on both hind flanks. The control group had free access to drinking water, the treated group received water supplemented with thiaproline. **A** The animals were weighed at regular intervals. Each point represents the mean weight of 9 animals ± SEM. **B** The water intake of animals was measured at regular intervals. Each point represents the mean water intake per mouse derived from 2 cages of 5 animals.

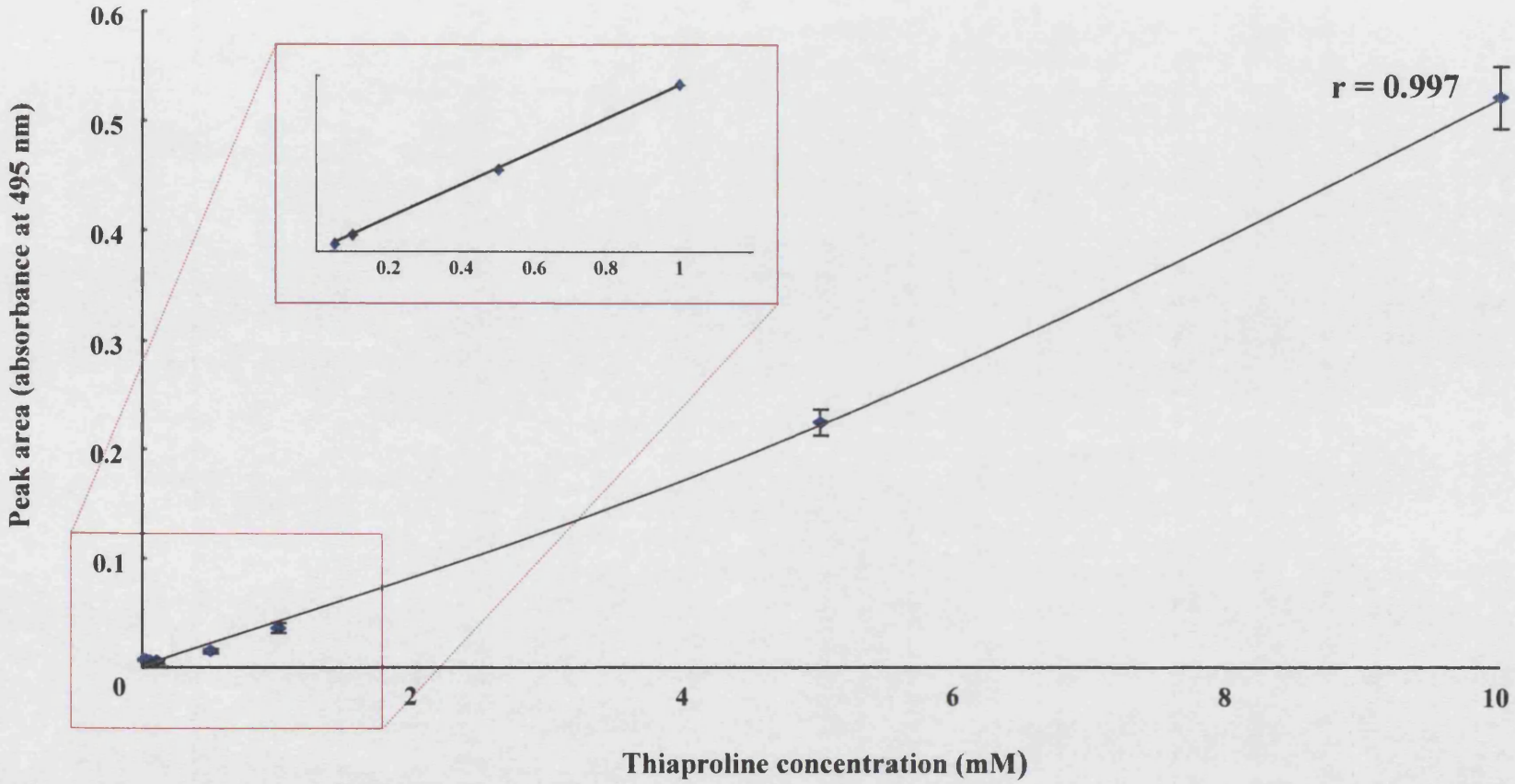


Figure 5.8 Thiaproline may be quantified by high performance liquid chromatography (HPLC). Thiaproline standard solutions (0.05 - 10 mM) were run through a HPLC system and the area of a peak eluting at approximately 7.8 minutes corresponding to thiaproline was measured. Highlighted section represents an enlarged area of graph demonstrating the ability to detect thiaproline from a concentration of 0.05 mM. Similar data was obtained from three independent experiments.

demonstrated a linear relationship between the peak area on the chromatogram and the concentration of thiaproline.

Figure 5.9 shows a chromatogram of plasma standards examined for thiaproline. A naturally occurring peak in unspiked human blood plasma (5.9B) was eluted at the same time as the thiaproline peak (figure 5.9C) which made quantification of low thiaproline concentrations impossible. The same problem was encountered when analysing blood plasma samples from control (figure 5.10A) and thiaproline treated mice (figure 5.10B). The elution conditions were modified several times (the buffer composition was altered to a higher percentage of acetonitrile and the running time for each sample through the column was increased) but this did not resolve the contaminating peak from the thiaproline peak.

5.3 Determination of thiaproline-treated and control tumour collagen content

The collagen content of thiaproline-treated and control tumours was assessed and represented as collagen content against tumour weight. The data from the 10 day tumours (figure 5.11A) showed that the thiaproline-treated tumours had a lower mass and contained a lower collagen content per tumour compared with control tumours. The correlation coefficient between tumour weight and collagen content was 0.97 demonstrating that an increase in tumour weight was associated with an increase in collagen content. Figure 5.11B demonstrates that thiaproline-treated tumours contained a lower concentration of collagen per mg of tumour weight than the control tumours ($p<0.001$).

5.4 Histology of tumour sections

Thiaproline-treated and control tumours were processed to paraffin wax and sections stained for collagen. Figure 5.12 demonstrates Martius scarlet blue staining of control and treated tumours at 10 and 18 days. The thick blue fibres apparent in the ECM correspond to deposited collagen fibrils and showed a similar distribution of collagen in both sets of tumours. Reticulin staining (figure 5.13) showed grey strands in the tumour stroma which demonstrated the presence of reticulin (collagen type I and type III and elastin) in a similar distribution in all tumour sets.

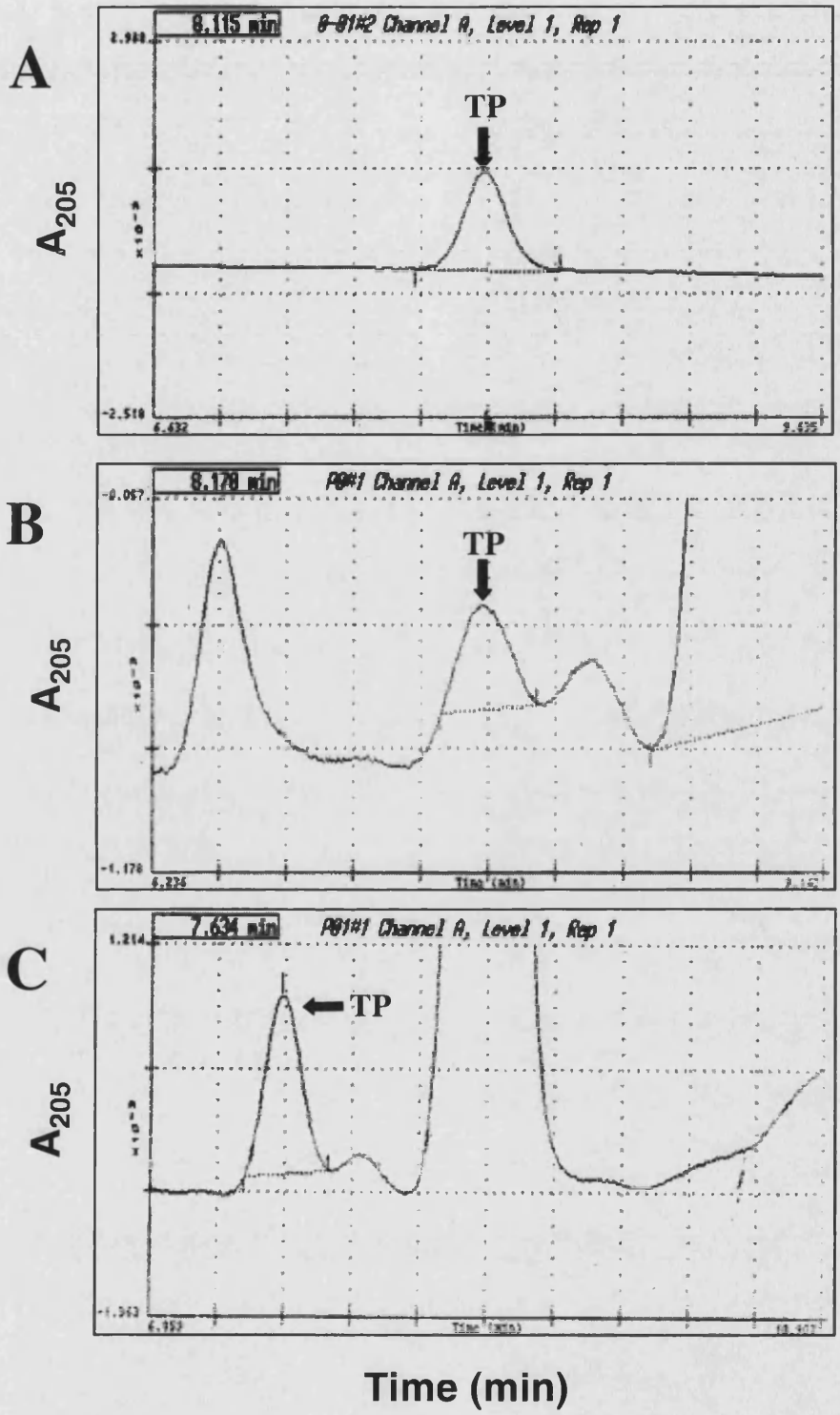


Figure 5.9 High performance liquid chromatograms of thiaproline standard and control/spiked human blood plasma. Thiaproline standard solution (**A;** 0.01 mM), human blood plasma (**B**) and thiaproline-spiked plasma (**C**; 0.01 mM) were run through a HPLC system. **TP** indicates the elution time for thiaproline.

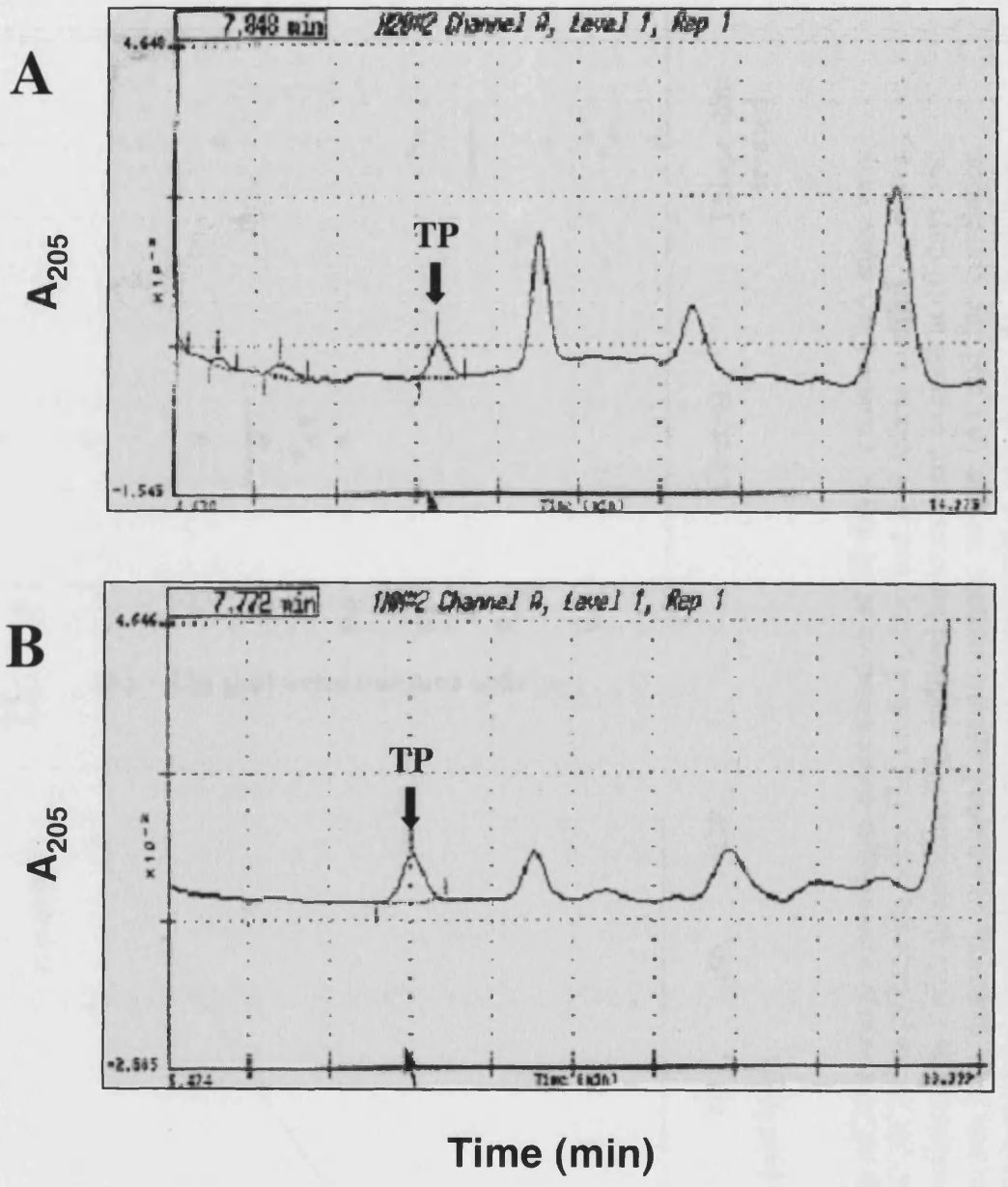


Figure 5.10 Chromatograms of mouse blood plasma demonstrated a naturally occurring peak masking thiaproline. Control mouse blood plasma (**A**) and thiaproline-treated mouse plasma (**B**) were processed on a HPLC system. **TP** indicates the elution time for thiaproline.

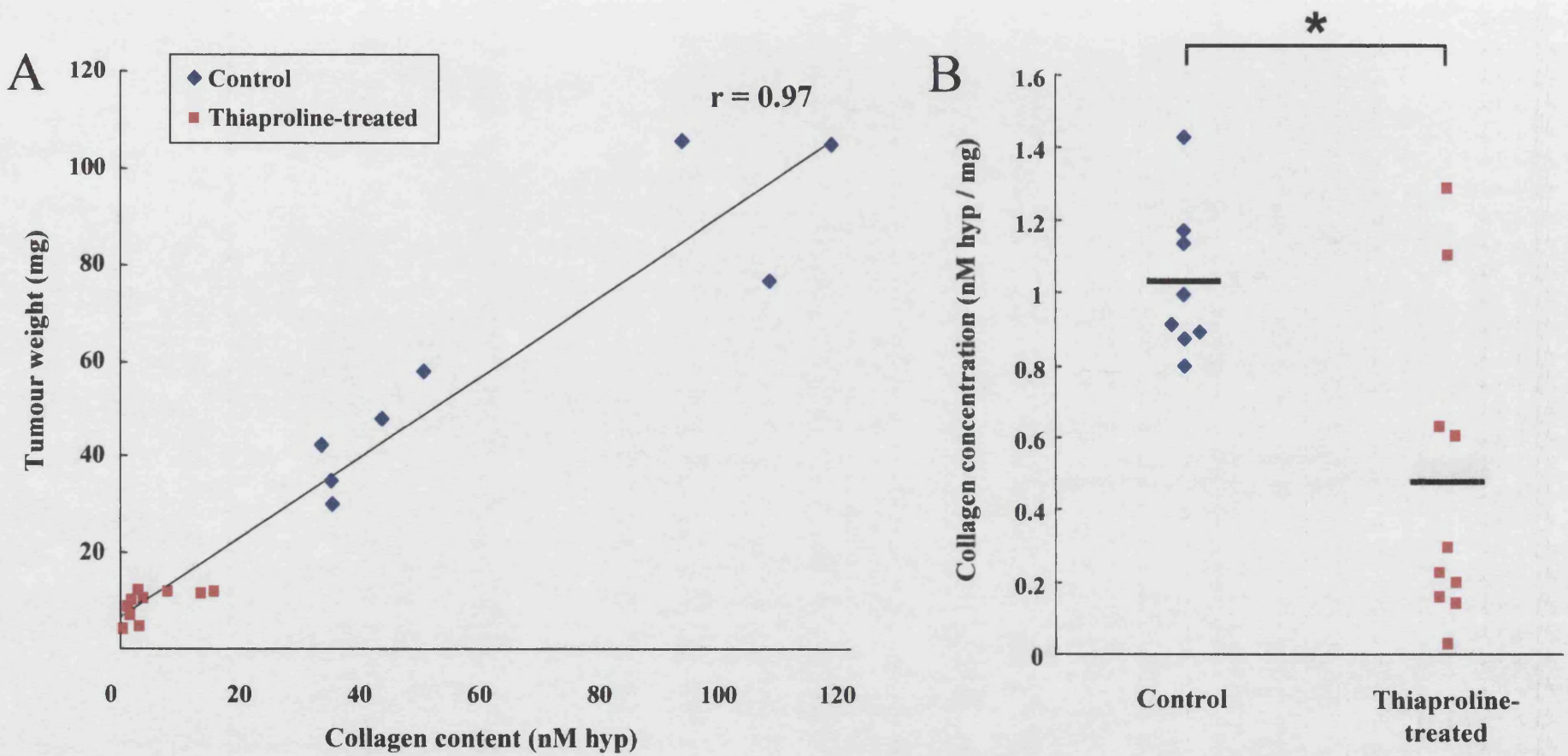


Figure 5.11 Thiaproline reduced AC29 tumour procollagen concentration at 10 days. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells. The control group had free access to drinking water, the treated group received water supplemented with thiaproline. The induced tumours were excised at 10 days and weighed. Tumour procollagen content was assessed and plotted against tumour weight (**A**) and the correlation coefficient (**r**) calculated. The concentration of procollagen per mg of tumour was also calculated (**B**). Each point represents an individual tumour. * denotes a significant difference of $p<0.01$ between thiaproline-treated and control tumour groups.

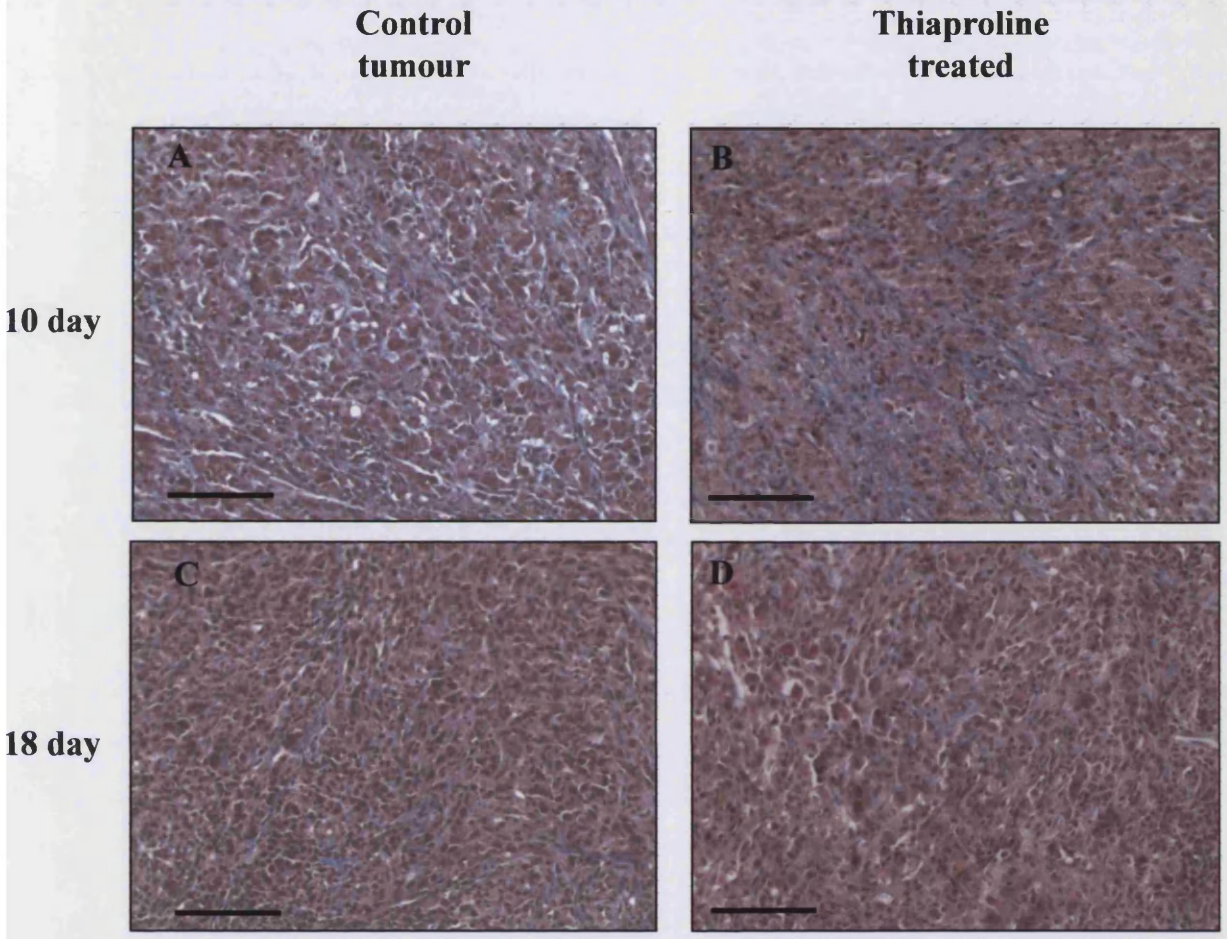


Figure 5.12 Martius scarlet blue staining revealed abundant collagen deposition in tumour sections. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells. The control group had free access to drinking water. The treated group received water supplemented with thiaproline. The induced tumours were excised at 10 and 18 days and processed to paraffin sections. Control tumour (**A**; 10 day, **C**; 18 day) and treated tumour (**B**; 10 day, **D**; 18 day) were stained with Martius scarlet blue. Bar represents 100 μm, original magnification x40.

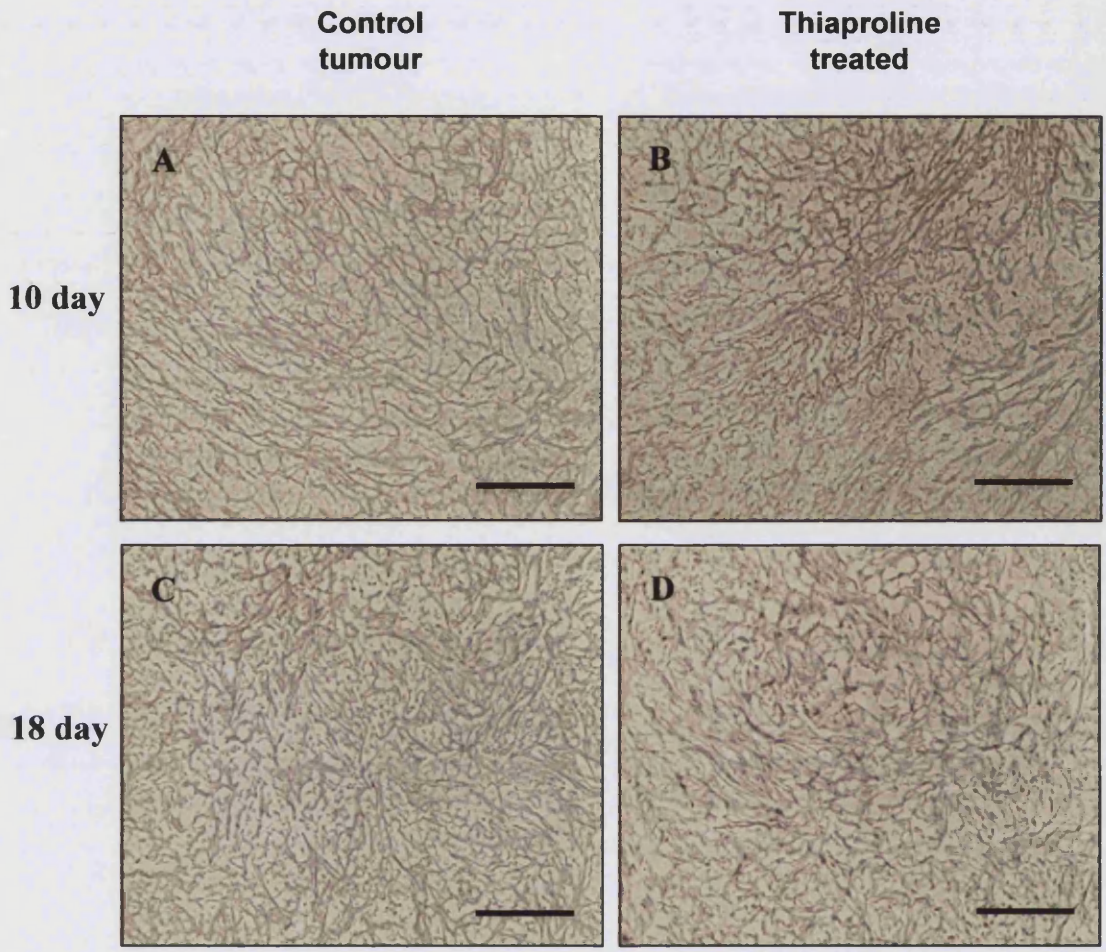


Figure 5.13 Reticulin staining demonstrated well defined reticular network in tumours. Thiaproline-treated AC29 tumours (section 2.7.3) were excised at 10 and 18 days and processed to paraffin sections. Control tumour (**A**; 10 day, **C**; 18 day) and treated tumour (**B**; 10 day, **D**; 18 day) were stained for reticulin (section 2.9.4). Bar represents 50 μm, original magnification x200.

Immunohistochemical staining for different collagen types revealed unique distribution in each case, although no obvious differences between the thiaproline-treated and control tumours were apparent at either time point. Collagen type I exhibited a fibrillar pattern in the ECM within the tumour (figure 5.14), collagen type III appeared to be much more diffuse with an amorphous distribution (figure 5.15) and basement membrane collagen type IV showed dense fibril-like staining (figure 5.16). All collagen types were present in the tumour capsule encircling the periphery of the tumour.

Haematoxylin and eosin staining of 10 day (figure 5.17) and 18 day (figure 5.18) tumour sections demonstrated an amorphous and heterogeneous cell population with a large nucleus to cytoplasmic ratio, characteristic of tumour cells. The majority of blood vessels were located in the periphery of the tumour. An inflammatory infiltrate (predominately macrophages) was also localised to the periphery, penetrating approximately 50 – 100 cell layers into the tumour, regardless of tumour size (figures 5.17 and 5.18). There were no observable differences in inflammatory cell or blood vessel number between the treated and control tumours.

5.5 Discussion

5.5.1 Murine models of MM tumour growth

The i.p. injection of AC29 cells into syngeneic mice is an established model of MM (Bielefeldt-Ohmann *et al.,* 1995a, Bielefeldt-Ohmann *et al.,* 1995b). In these studies the extent of tumourigenicity was assessed using scoring methods examining variables such as body weight, behaviour, and physical appearance over a period of up to 3 months. However, for the current study, at 18 days no observable symptoms of i.p. tumour inoculation were observed and so tumour extraction and weight were used (Lukacs *et al.,* 1999). The i.p. tumours grew as an inseparable aggregation of tumour bulk and abdominal organs and thus the entire abdomen contents were removed and weighed (figure 5.1). Biological variation between abdominal weights in the control group mask the comparatively smaller differences in weight of the induced tumours and therefore made this model unsuitable for studying the effect of thiaproline on MM tumour growth. However, there was a trend towards a decrease with thiaproline treatment, the treated group having an approximate 5 % decrease in mean abdominal weight compared to the injected control group.

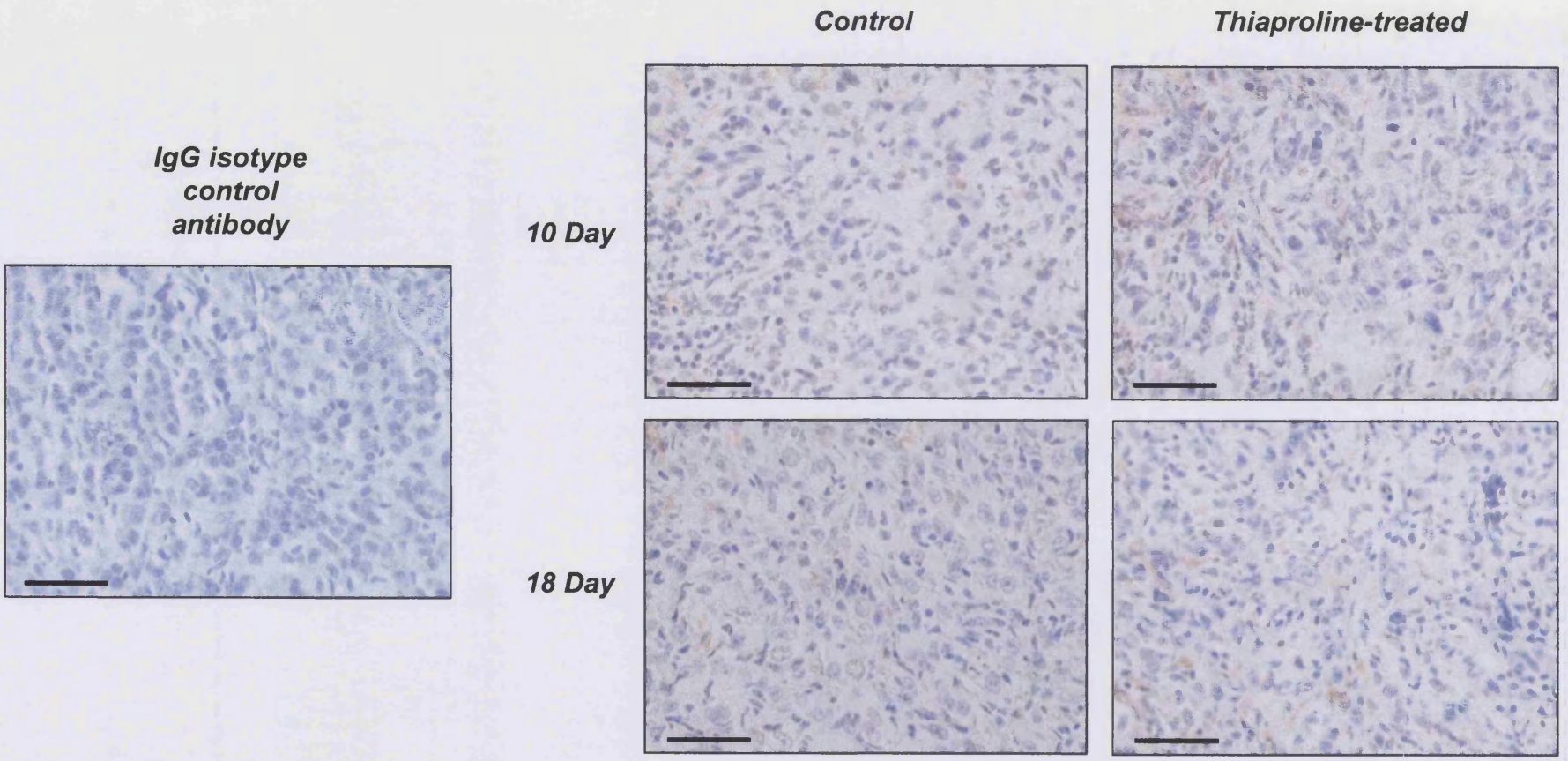


Figure 5.14 Immunohistochemistry revealed fibrillar collagen type I distribution in AC29 solid tumours. Thiaproline-treated induced AC29 tumours (section 2.7.3) were excised at 10 and 18 days, processed to paraffin sections and stained with a collagen type I specific antibody (section 2.9.6). Bar represents 25 μm, original magnification x200.

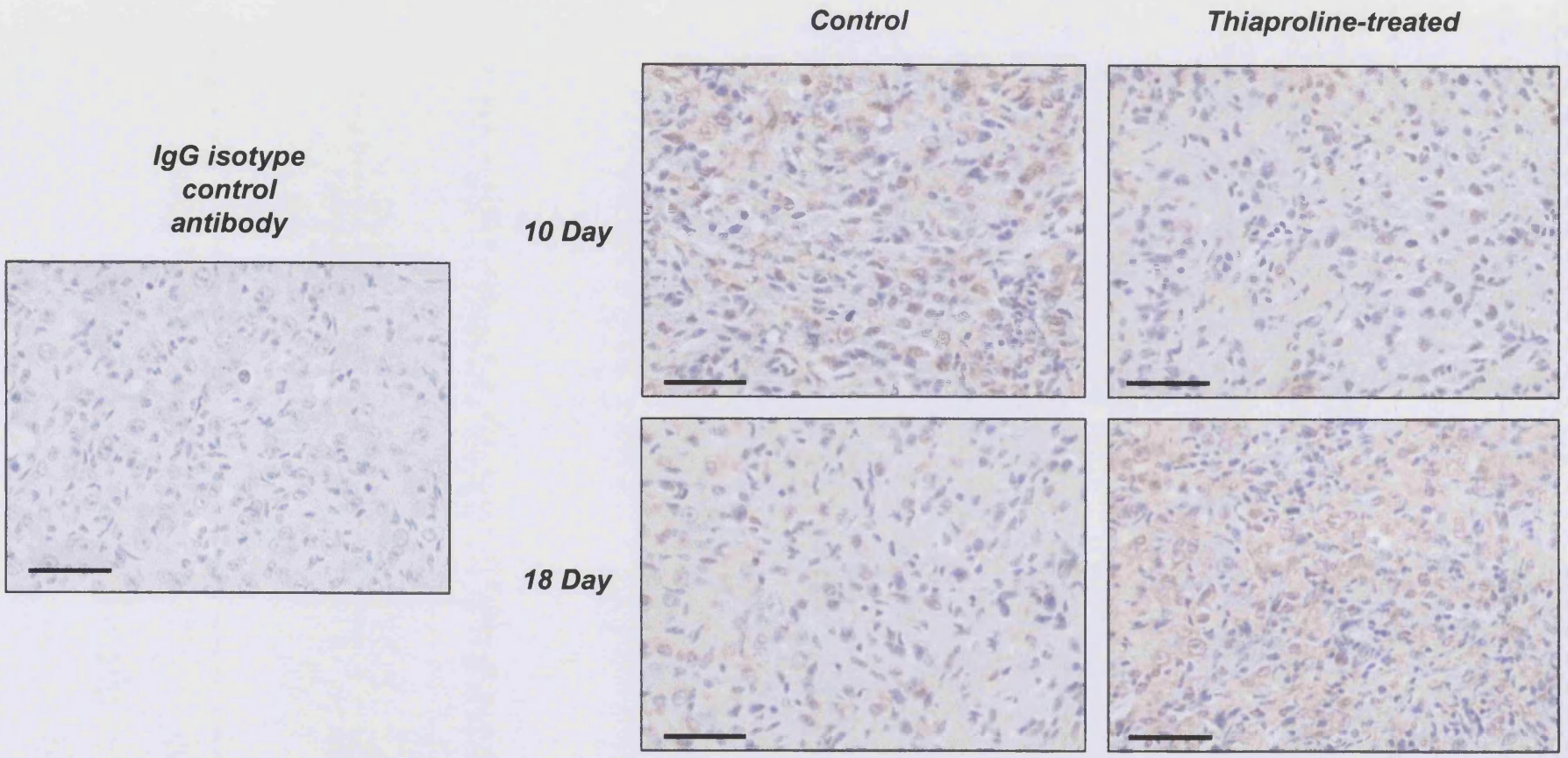


Figure 5.15 Immunohistochemistry revealed a large distribution of fibrillar collagen type III in tumour sections. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells. The control group had free access to drinking water. The treated group received water supplemented with thiaproline. The induced tumours were excised at 10 and 18 days, processed to paraffin sections and stained with a collagen type III antibody (section 2.9.6). Bar represents 25 μm, original magnification x100.

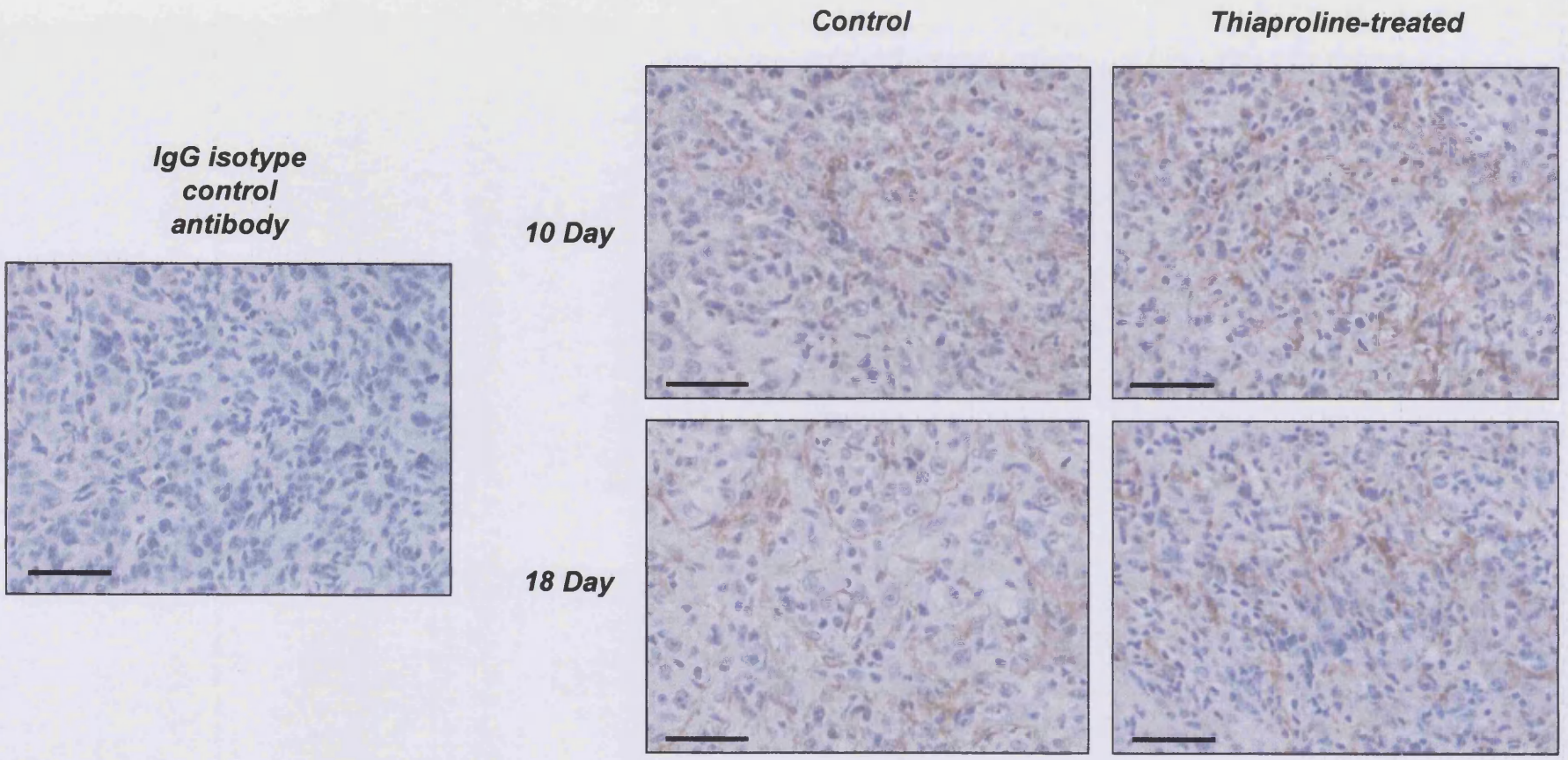


Figure 5.16 Immunohistochemistry showed the presence of basement membrane collagen type IV in tumours. Thiaproline-treated AC29 solid tumours (section 2.7.3) were excised at 10 and 18 days, processed to paraffin sections and stained with a collagen type IV specific antibody (section 2.9.6). Bar represents 25 μm, original magnification x100.

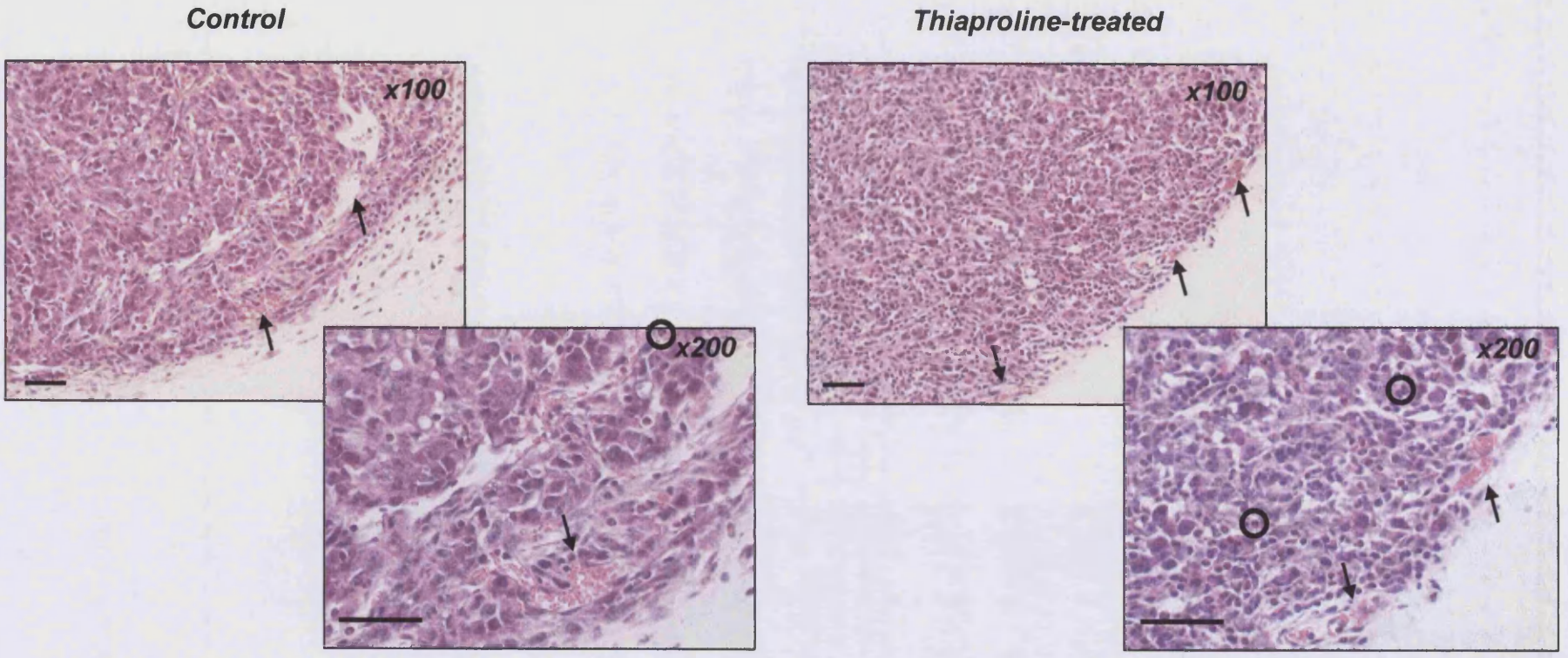


Figure 5.17 Haematoxylin and eosin staining revealed blood vessels close to the periphery of tumours at 10 days. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells. The control group had free access to drinking water. The treated group received water supplemented with thiaproline. The induced tumours were excised at 10 days, processed to paraffin sections and treated with Haematoxylin and eosin stain (section 2.9.5). Arrows mark areas of vasculature, circles highlight macrophages. Bar represents 25 μm.

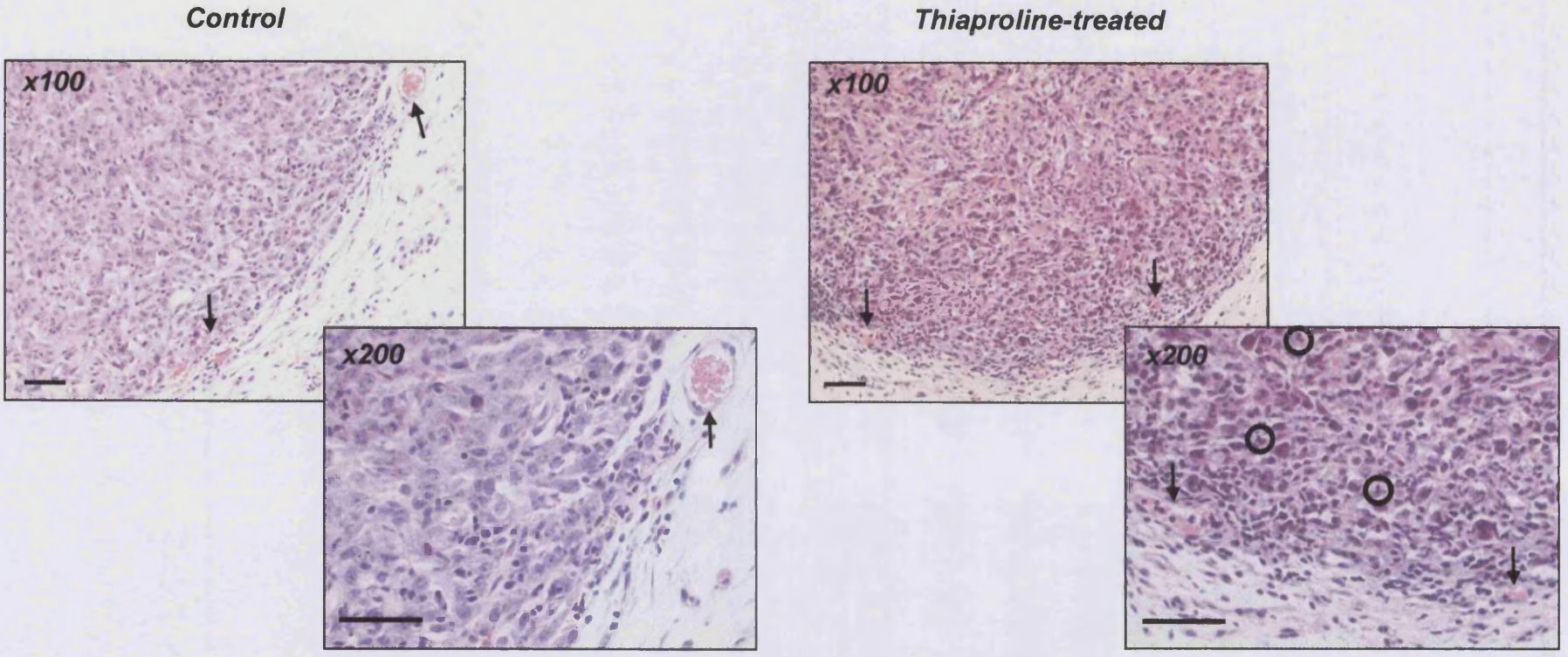


Figure 5.18 Haematoxylin and eosin staining showed established vasculature in 18 day tumour sections. Thiaproline-treated induced AC29 tumours (section 2.7.3) were excised at 18 days, processed to paraffin sections and treated with Haematoxylin and eosin stain (section 2.9.5). Arrows indicate vessels, circles surround macrophages Bar represents 25 μm.

The s.c. injection of MM cells into syngeneic mice is an alternative model of MM growth (Davis *et al.*, 1992). This model has been demonstrated to be consistent with the human disease in almost every aspect, in terms of histology, ultrastructure, growth, morphology and tumourigenicity. The s.c. model was more amenable for tumour studies than the i.p. model as s.c. induction of MM enabled the assessment of tumour growth externally without the need to sacrifice the animal, as well as generating a single defined tumour mass which was convenient for analysis.

Tumour growth was initially measured by obtaining two perpendicular measurements on the excised tumour using microcallipers at the time of sacrifice. Figure 5.2 shows that AC29 had the fastest rate of growth between 18 and 25 days, producing larger tumours than AB1 or AB22. Other studies assessed rate of growth by measuring tumour dimensions whilst still *in vivo* through the skin (Hwang *et al.*, 1995, Leong *et al.*, 1997, Caminschi *et al.*, 1999). However, these methods could be considered to be imprecise. Skin thickness influenced size measurements, and given the irregular nodular shape of the tumours (figure 5.3) it was not an accurate reflection of tumour size using measurements in two dimensions and calculating volume. Due to these concerns weight was measured as an assessment of tumour growth (figure 5.4). The surgical removal and weighing of the tumours allowed a more precise measurement of tumour growth from an earlier time-point than measuring tumour dimensions externally through the skin. Subsequent *in vivo* studies used weight as a measure of tumour growth.

5.5.2 Administration of thiaproline in vivo delayed tumour growth

Previous *in vivo* studies have shown a range of doses of thiaproline that are non-toxic when given orally to mice. Lubec *et al.* (1994) observed a reduction in glomerular basement membrane thickening in the diabetic db/db mouse following treatment with thiaproline at 30 mg/kg/day for 12 weeks. Correa and associates (1999), studying the effect of thiaproline on macrophage function, fed mice thiaproline at 0.1% w/w in food for 5 weeks without any toxicity. In this study, mean animal body weight was measured over the course of thiaproline treatment with no difference seen between the treated and untreated groups (figure 5.7A). Body weight increased as expected from the supplier's growth curves. To demonstrate that thiaproline was not affecting water intake, and that animals in treatment groups received equal doses, the volume of

drinking water was measured daily (figure 5.7B). There was no significant difference in the mean daily water consumption per animal between the treated and untreated groups. No observable difference in the behaviour or appearance of treated and untreated animals was noticed, confirming previous studies (Lubec *et al.*, 1994).

In order to be confident that thiaproline was reaching the tumours in the animal model, an attempt was made to measure the circulating level of thiaproline in the blood plasma of control and treated animals. Figure 5.8 demonstrates that thiaproline could be measured from a concentration of 0.05 mM in standard solutions, and that thiaproline concentration was strongly correlated with peak area. However, when measuring thiaproline in extracted plasma, there was an unidentified peak present at the same position as thiaproline in the standards (figure 5.9). This unidentified peak masked the thiaproline peak making quantification impossible.

Pine *et al.* (1983) previously demonstrated that thiaproline could be detected in mouse blood plasma at a circulating concentration of 77 nmol/ml when given orally in drinking water at 8 mg/ml. At lower doses they were unable to detect circulating thiaproline. In the current study, thiaproline was given to mice at a dose of 0.4 mg/ml of drinking water, equivalent to a concentration of 1mM, which has been demonstrated to be effective in reducing collagen production (figure 4.7) and cell proliferation (figure 4.9) whilst not being toxic (figures 4.3 – 4.6). Therefore, at this concentration thiaproline was too dilute to be detected and measured in blood plasma using the method of Pine and colleagues.

A possible solution to detect blood plasma thiaproline would be to employ the use of high-resolution ^{1}H NMR spectroscopy, which is able to detect compounds at parts per million sensitivity, which equates to pM concentrations. Recent publications investigating metabonomics demonstrated the sensitivity of this system using plasma and urine samples (Tate *et al.*, 2001; Sidelmann *et al.*, 2001; Nicholls *et al.*, 2001).

In this study at 10 days, oral administration of thiaproline at a dose of 100 mg/kg/day significantly lowered AC29 median tumour weight by approximately 85% compared with the control group (figure 5.5). There was no overlap of tumour weights from one group to another, and within the thiaproline treated group there was a minimal spread

of weight. Collagen analysis of the tumours revealed that there was a significantly lower concentration of collagen per tumour in the thiaproline treated group compared with the control group (figure 5.11B). When collagen content was expressed with respect to tumour weight there was a strong correlation between tumour weight and collagen content (figure 5.11A). This showed that an increase in tumour weight was associated with an increase in collagen content and that the greater tumour mass seen in the control group was not only due to an increase in tumour cells or fluid retention. These data demonstrated that the use of thiaproline resulted in significantly smaller tumours with a significantly lower concentration of collagen, whereas in untreated animals, tumours were larger with a higher concentration of collagen.

The *in vitro* studies from chapter 4 demonstrated that AC29 had an enhanced rate of proliferation on a collagen type I substrate (figure 4.1), that thiaproline inhibited AC29 collagen production (figure 4.7) and that thiaproline also reduced AC29 proliferation (figure 4.9). These data are consistent with the hypothesis that collagen production stimulates MM proliferation. Together, the *in vitro* (chapter 4) and *in vivo* data clearly demonstrate that thiaproline inhibited collagen production, which correlated with a reduced rate of tumour growth up to 10 days post transplantation.

At 18 days there was no significant difference in tumour weight between the treated and untreated groups (figure 5.6). Therefore, for the tumour size to be the same in both groups either the treated tumours increased their rate of growth, growth of control tumours slowed, or a combination of both occurred. The reasons for this are unclear. It is possible that between 10 and 18 days the control tumours reached a peak in their rate of growth, with the tumours being unable to support a higher rate of growth due to insufficient vasculature. Areas of necrosis at the core of several tumours were seen at 10 and 18 days (data not shown), these could have been due to insufficient vasculature leading to cell death. However, the median tumour weight of the control group approximately doubled between 10 to 18 days showing that an attenuation of control tumour growth did not occur. It is also possible that at 10 days in the treated group there were insufficient collagen producing cells to sustain a rapid rate of growth, or that a critical amount of collagen was required to be produced before the rate of growth increased. During 10 to 18 days a critical number of cells may have been reached that could produce the required quantities of matrix

components and cytokines allowing the tumour to grow at a faster rate. The integrin family of receptors mediates the interaction between cells and the ECM. After being activated by an ECM ligand, integrins stimulate intracellular signalling and can promote cell-cell aggregation and adhesion to ECM components (Sethi et al., 1999). The lower concentration of collagen at 10 days in the thiaproline treated group potentially provides less ligand for the integrins to bind resulting in slower growth. This may be overcome as sufficient ECM is produced between 10 - 18 days.

Administration of higher doses of thiaproline may have had a greater effect in delaying tumour growth, however there was concern regarding its *in vivo* toxicity. Toxicity has been observed at different concentrations in various species when given orally and intraperitoneally (Nasca *et al.*, 1981, Newman *et al.*, 1980). It has been reported that the most toxic route of thiaproline administration is that given orally (Gosalvez *et al.*, 1980). Therefore doses of thiaproline above the 100 mg/kg/day reported here were not investigated.

A similar delay in tumour growth to that demonstrated in this study has been observed in several other *in vivo* studies of mesothelioma. Leong *et al.* (1994) have shown that AC29 cells transfected with allogenic MHC genes were able to retard, but not inhibit, the growth of parental AC29 cells injected into the same animal. AC29 cells transfected with the T-cell costimulator B7-1 had a delayed ability to grow *in vivo*, although all eventually formed tumours (Leong *et al.*, 1996). Mukherjee *et al.* (2001) established that a combination of surgical debulking and transfection of AC29 cells with B7-1 or GM-CSF resulted in a delay of tumour growth.

Fitzpatrick *et al.* (1994) and Marzo *et al.* (1997) demonstrated that antisense oligonucleotides to TGF-β_2 delay AC29 tumour development compared to controls. However, once the treated tumours became palpable they exhibited similar growth kinetics compared to the control groups. A possible explanation for this delay in tumour growth at earlier time points and subsequent 'catch up' growth in the treated group at a later stage may be due to the incomplete suppression of TGF-β_2 (Marzo *et al.*, 1997). At early stages of tumour growth the level of inhibition may be sufficient to restrict growth, however once the tumour reaches a certain size this level of

inhibition is no longer strong enough to slow growth due to the greater cell number within the tumour. This thesis has demonstrated that at a concentration of 1mM thiaproline inhibits collagen production by approximately 20% *in vitro* (figure 4.8) and other studies have demonstrated that similar concentrations of thiaproline reduce collagen accumulation *in vivo* (Lubec *et al.*, 1994, 1997). At a concentration of 1mM thiaproline decreased tumour size compared to untreated controls at 10 days (figure 5.5). However, the remaining 80% of collagen production may have been sufficient to promote tumour growth between 10 – 18 days resulting in the similar sized thiaproline-treated and control tumours observed (figure 5.6).

A delay in tumour growth has been apparent in several clinical studies of MM where chemoimmunotherapeutic methods have been used to try and improve patient survival. Trials involving the use of interferon-α in combination with other chemotherapy agents (Upham *et al.*, 1993, Ardizzoni *et al.*, 1994, Fizazi *et al.*, 2000) increase the median survival of patients marginally. These studies show partial responses in some patients suggesting that these treatments are delaying tumour growth slightly, but that the disease is still fatal.

Martius scarlet blue (figure 5.12) and haematoxylin and eosin staining (figures 5.17 and 5.18) demonstrated an amorphous and heterogeneous cell population with a large nucleus to cytoplasm ratio, characteristic of malignant cells. Histological assessment of tumours did not show differences between treated and control sections (figures 5.12 – 5.18). There was a very similar pattern of matrix distribution and intensity of staining for collagen and reticulin, both of which contributed to the body of the tumour and occurred as part of the surrounding tumour capsule. Reticulin staining (figure 5.13) demonstrated less mature reticulin around the periphery of the tumours suggesting that the matrix around the tumour edges was laid down later and tumour growth occurred outwards.

The apparently similar collagen distribution shown in figures 5.14 – 5.16 is contradictory to figure 5.11B which demonstrated a lower concentration of collagen in the thiaproline treated group when compared with the control group at 10 days. The difference could be attributed to the fact that histology is a 2-dimensional

representation of 3-dimensional tissue and there could have been uneven collagen deposition across the tumour mass. Collagen analysis by HPLC sampled the entire tumour collagen content and would therefore give a better indication of the actual overall collagen content.

Specific immunohistochemical staining revealed the presence of fibrillar collagens type I and type III (figures 5.14 and 5.15). Collagen type I had a fibrillar network pattern, collagen type III a more amorphous distribution than type I. However, the collagen type III antibody exhibits a 70% cross-reactivity with type I collagen (manufacturer's data) and therefore shows the distribution of collagen type I and type III. The basement membrane collagen type IV was also found distributed in the tumour stroma in a dense fibrillar pattern (figure 5.16). This has also been observed in gastric and bile duct carcinomas immunohistochemically and by *in situ* hybridisation (Chen *et al.*, 2000**b**). The authors suggest that stromal collagen type IV deposition may increase the extent of desmoplasia in these tumours. MM has been shown to adhere and migrate to collagen type IV (Klominek *et al.*, 1997), and collagen type IV has been implicated in breast carcinoma growth (Lewko *et al.*, 1981). Reduction of tumour growth by proline analogues inhibiting collagen production has been found to be most effective in tumours that synthesise collagen type IV (Klohs *et al.*, 1985). The data presented here demonstrating enhanced proliferation of MM cells on collagen substrates (figures 4.1 and 4.2), the abundance of collagen in MM, and the inhibition of tumour growth with thiaproline supports a role for collagen in MM tumour growth. The decrease in proliferation observed with thiaproline (figure 4.9), an inhibitor of collagen production (figure 4.7), provides further evidence for the importance of collagen in MM growth.

Haematoxylin and eosin staining (figures 5.17 – 5.18) revealed that the majority of blood vessels were located close to the periphery of the tumour. There were no apparent differences in the number of vessels or inflammatory cell numbers between groups.

5.5.3 Non-collagen related effects of thiaproline

Several studies suggest that thiaproline has other effects apart from inhibition of collagen production, and these may influence tumour growth. The inhibition of

angiogenesis and the regression of growing capillaries in the chick chorioallantoic membrane were observed with thiaproline treatment (Ingber and Folkman, 1988). Angiogenesis is essential for tumour growth beyond 1 – 2 mm in diameter and there are studies which demonstrate that inhibiting angiogenesis may be a suitable strategy for treating cancer growth (Riedel and Hormann, 2001, Rozic *et al.*, 2001, Katzenstein *et al.*, 2001). Therefore, thiaproline may have at least partly delayed tumour growth through inhibition of angiogenesis. However, histologically there didn't appear to be any difference in the number or structure of vessels within the tumours (figures 5.17 – 5.18) although further studies with markers specific to endothelial cells to enable quantification are required to confirm this.

The ability of cancer to evade the host immune system allows unchecked tumour growth. A possible control of MM would be to stimulate local and systemic anti-tumour immune responses. Bielefeldt-Ohmann *et al.* (1995a) have shown that the treatment of MM bearing mice with recombinant human interferon-α (rhuINFα) curtailed the clinical symptoms of the disease accompanied by improved lymphocyte activity and an enhanced number of tumour-infiltrating lymphocytes and macrophages. Therapy with rhuINFα has also been demonstrated to have stimulatory effects on immune cells with a decreased rate of MM tumour growth *in vivo* (Bielefeldt-Ohmann *et al.* 1995b).

There are several studies suggesting that murine immune functions can be improved by thiaproline, both *in vitro* and *in vivo*. The lymphoproliferative response, lymphocyte motility and the natural killer activity of leukocytes in mice were all significantly stimulated by ingested thiaproline (De la Fuente *et al.*, 1993, 1998). It is also suggested that thiaproline may enhance murine macrophage function (Correa *et al.*, 1999). As the range of concentrations of thiaproline used in these studies were similar to those used in this study, an improvement in immune function by thiaproline may have contributed to the delayed tumour growth at 10 days. However, haematoxylin and eosin staining revealed no differences in inflammatory cell influx between treated and control sections at 10 days (figures 5.17 – 5.18). Further studies to quantify inflammatory cells are required to confirm this.

Thiaproline given orally is metabolised in the liver to cysteine and formaldehyde (Grier *et al.*, 1984, Zhao *et al.*, 1995). Formaldehyde release leads to cytotoxic damage, which would limit tumour growth. There have been several reports documenting the effect of aldehydes on inhibiting tumour growth (Schauenstein and Esterbauer, 1978, Gescher *et al.*, 1982). However, ingested thiaproline is probably partially removed in the portal circulation (Pine *et al.*, 1983) and at the dose used in this study, mice exhibited no toxic or behavioural problems associated with aldehyde poisoning.

The non-collagen related effects of thiaproline described above may have contributed to the lower tumour mass seen in the thiaproline-treated group. However, collagen analysis revealed a lower concentration of collagen per mg of tumour in the thiaproline-treated group compared with the control group (figure 5.11B). This observation suggests that despite the other effects of thiaproline, decreased collagen production was associated with delayed tumour growth. This is consistent with the observation in the previous chapter that inhibition of collagen production with thiaproline decreased AC29 cell proliferation (figure 4.9).

5.6 Summary and conclusions

The experiments described in this and the previous chapter demonstrate that MM cells produce collagen and furthermore proliferate at a significantly faster rate when grown on a collagen matrix. Inhibition of collagen production with thiaproline significantly reduced cell proliferation *in vitro* and tumour weight and collagen concentration at 10 days but not 18 days of treatment *in vivo*. These novel findings demonstrate that inhibition of collagen production delays MM tumour growth. The use of thiaproline in the treatment of human MM would not be feasible due to the cytotoxic nature of this compound. However, this study has clearly shown that therapeutic agents capable of inhibiting collagen production at non-toxic doses could be effective in the treatment of MM. A multimodal approach combining surgical debulking of the tumour followed by the use of an inhibitor of collagen production may prove to be a viable therapeutic strategy for the treatment of this disease.

This thesis has demonstrated that collagen production is potently stimulated by TGF-β (table 3.1) and that TGF-β is at elevated levels in MM (figure 3.3). The effect of the different TGF-β isoforms on MM collagen production and cell proliferation have not previously been determined. Therefore, the next chapter investigates the role of different exogenous TGF-β isoforms on MM cell proliferation and the effect of specifically neutralising TGF-β isoforms on proliferation and collagen production *in vitro*.

Chapter Six

The role of exogenous and endogenous Transforming Growth Factor-β_{1-3} on mesothelioma cell proliferation and collagen production in vitro

6.1 Introduction

Chapters 4 and 5 demonstrated that inhibition of procollagen production in MM cells reduced their proliferation *in vitro* and delayed tumour growth *in vivo*, suggesting an important role for collagen in MM growth. Also, levels of transforming growth factor-beta (TGF-β_{1-3}), key regulators of cell proliferation and potent stimulators of collagen production, are increased in MM. Compared to normal mesothelial cells, MM produces elevated levels of TGF-β at the RNA and protein level (Fitzpatrick *et al.*, 1994, Kuwahara *et al.*, 2001), and in pleural effusions from MM patients TGF-β is present at 3 – 6 times the levels found in other primary lung cancers (Maeda *et al.*, 1994). Increased levels of TGF-β have also been demonstrated in MM cells compared with normal mesothelial cells in this study, and exogenous TGF-β_1 shown to stimulate MM cell procollagen production in a dose dependent fashion *in vitro* (chapter 3). The following two chapters test the hypothesis that autocrine TGF-β production by MM promotes cell proliferation and tumour growth through the enhancement of collagen production. A comparison of the specific endogenous TGF-β isoforms produced by normal mesothelial cells and MM has not previously been performed and TGF-β isoform specific effects on MM cell proliferation and collagen production have not been assessed. The aims of this chapter are to:

1. determine the effect of exogenous TGF-β isoforms on MM cell proliferation,
2. quantitate TGF-β isoform production at the protein level,
3. and examine the effect of inhibiting specific TGF-β isoforms on cell proliferation and procollagen production.

6.2 The effect of exogenous TGF-β_1, -β_2 and -β_3 on mesothelioma cell proliferation

DNA synthesis in AC29 cells in response to 24 hr incubation with a range of concentrations of TGF-β_1, -β_2 and -β_3 (0 – 10 ng/ml) was assessed by ^{3}H-TdR incorporation studies. Data was presented as percentage change from the medium control (figure 6.1). All three isoforms of TGF-β had a similar effect, reducing thymidine incorporation with increasing concentrations of TGF-β in a dose-dependant manner. At a concentration of 100 pg/ml TGF-β_1 significantly decreased AC29 thymidine incorporation compared with the medium control by approximately 15%

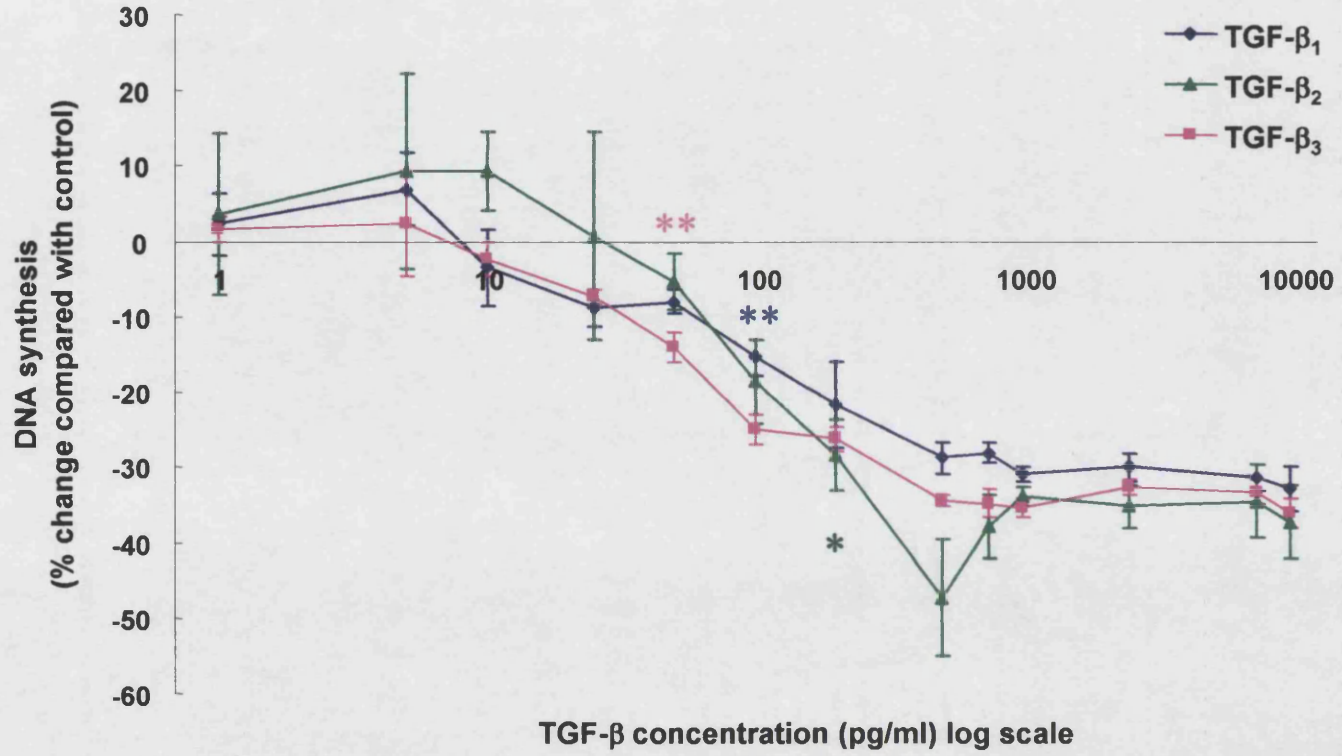


Figure 6.1 Exogenous TGF-β_1, -β_2 and -β_3 inhibited AC29 DNA synthesis. AC29 cells were incubated with increasing concentrations of TGF-β_{1-3} for 24 hr and DNA synthesis measured via tritiated thymidine incorporation. Data are expressed as percent change from medium control. *$p<0.05$, **$p<0.01$ vs. medium control. The results are representative of three repeat experiments.

(TGF-β_1-treated 26380 ± 730 d.p.m. / well, medium control 36980 ± 530 d.p.m. / well; $p<0.01$). Incubation with 200 pg/ml TGF-β_2 significantly reduced thymidine incorporation by approximately 30% (TGF-β_2-treated 18750 ± 1460 d.p.m / well, medium control 26200 ± 1650 d.p.m. / well; $p<0.05$). Thymidine incorporation was also reduced by approximately 15% with 50 pg/ml TGF-β_3 (TGF-β_3-treated 52660 ± 1190 d.p.m / well, medium control 61200 ± 1140 d.p.m. / well; $p<0.01$). Further inhibition of thymidine incorporation occurred with increasing concentrations of all TGF-β isoforms, with a maximal inhibitory effect seen at a concentration of approximately 1 ng/ml TGF-β.

6.3 Characterisation of isoform-specific TGF-β neutralising antibodies

To examine the amount of TGF-β protein secreted by cell lines into conditioned medium a panel of TGF-β antibodies were firstly characterised for their potency and selectivity towards the different TGF-β isoforms. The ability of the antibodies to neutralise 0.1 ng / ml TGF-β_1, -β_2 and -β_3 was assessed using a MLEC TGF-β bioassay. All of the antibodies were used at the manufacturers recommended concentrations.

The effectiveness of the R & D Systems TGF-β antibodies in neutralising TGF-β_1, -β_2 and -β_3 are shown in figure 6.2. The pan-specific TGF-β antibody was extremely potent, completely neutralising the activities of all three TGF-β isoforms, whilst the control antibodies had minimal effects. The TGF-β_1 and TGF-β_2 neutralising antibodies specifically blocked their respective isoforms with minor effects on the other TGF-β isoforms. TGF-β_1 antibody reduced TGF-β_1 activity by 100%, TGF-β_2 neutralising antibody decreased the activity of TGF-β_2 by 95%. The TGF-β_3 antibody neutralised TGF-β_3 activity by 95% compared to the control antibody. However, cross-reactivity was observed (as stated on the manufacturer's datasheet), as the TGF-β_3 antibody also decreased TGF-β_2 activity by approximately 65% compared to the antibody control.

Neutralising antibodies against TGF-β isoforms supplied by Cambridge Antibody Technology (CAT) for *in vivo* studies (chapter 7) were also characterised (figure 6.3).

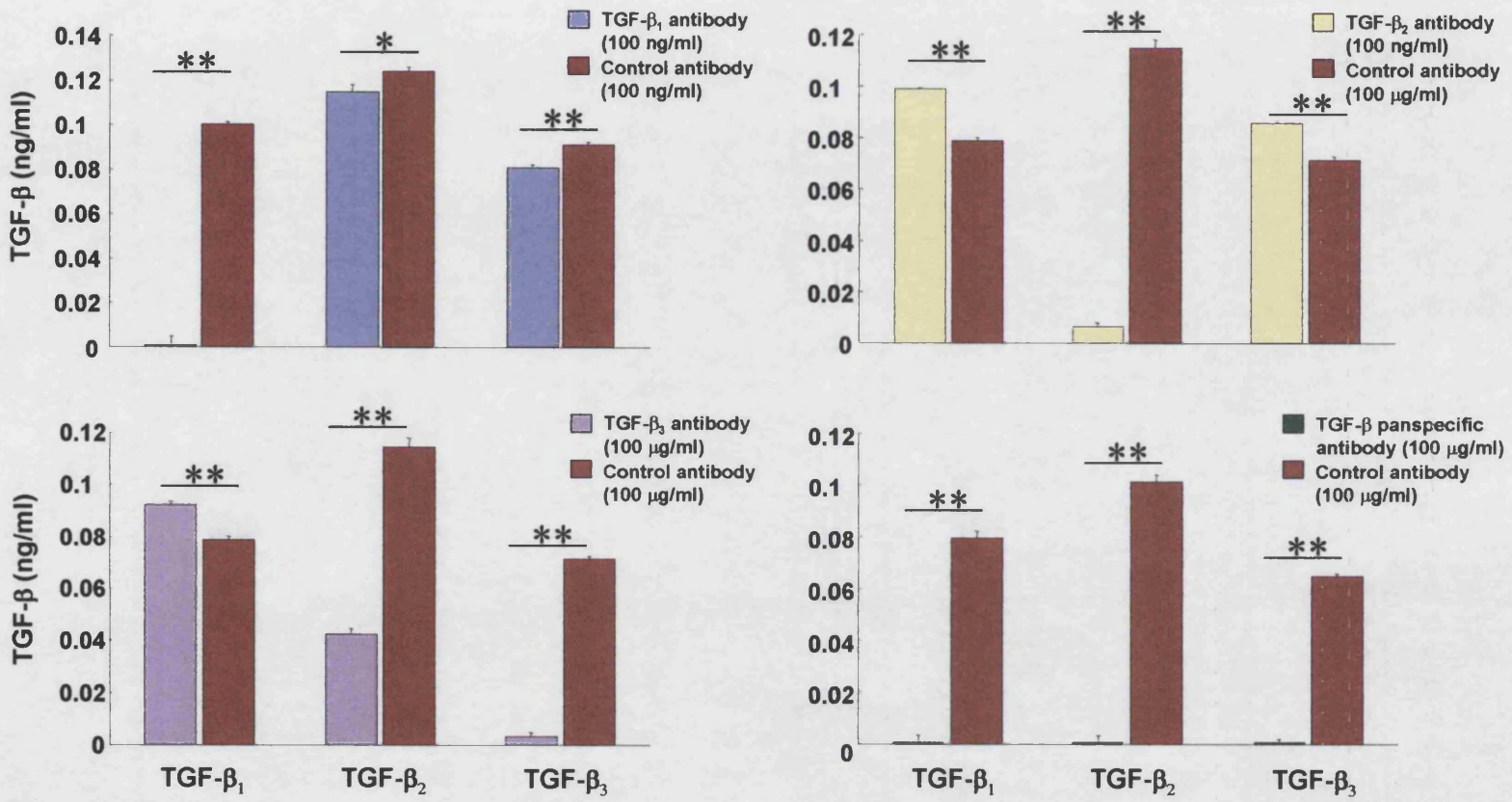


Figure 6.2 Characterisation of R&D Systems TGF-β antibodies. The efficacy of the TGF-β antibodies to neutralise 0.1 ng/ml TGF-β_{1-3} was assessed in a highly sensitive TGF-β bioassay (section 2.6.2). Each bar represents the mean ± SEM for six replicates. The antibodies were used at concentrations recommended by the manufacturer. *$p<0.05$, **$p<0.001$. Data are representative of two repeat experiments.

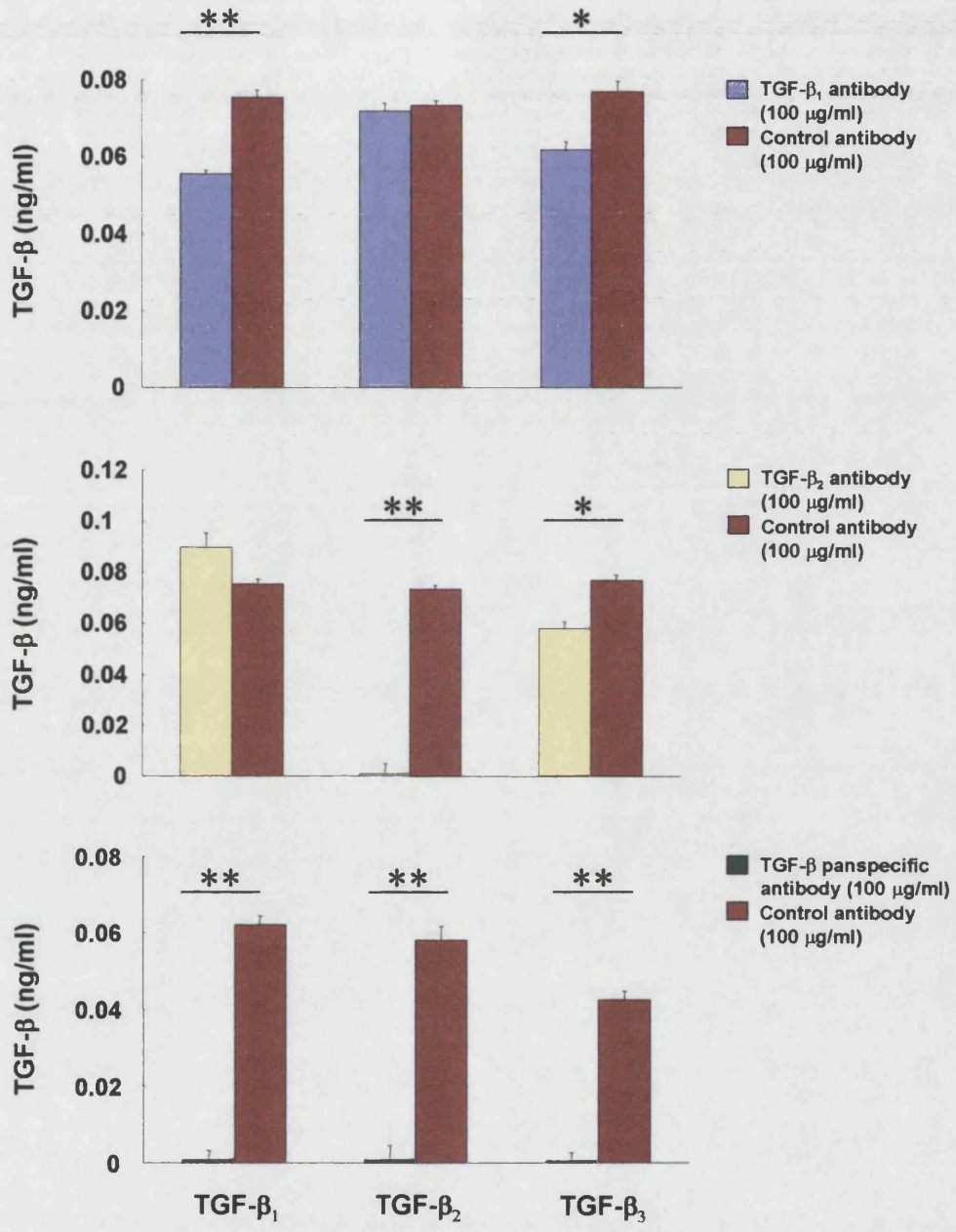


Figure 6.3 Characterisation of Cambridge Antibody Technology TGF-β antibodies. The specificity and potency of the CAT TGF-β antibodies to neutralise 0.1 ng/ml TGF-β_{1-3} was assessed using the MLEC TGF-β bioassay (section 2.6.2). Each bar represents the mean ± SEM for six replicates. The antibodies were used at concentrations recommended by the manufacturer. *$p<0.01$, **$p<0.001$. Data are representative of two repeat experiments.

The pan-specific TGF-β antibody was also highly potent, with 100% neutralisation of all three TGF-β isoforms. The TGF-β_1 antibody exhibited poor neutralisation of TGF-β_1, reducing activity by approximately 25% compared with the control antibody. There was a similar degree of cross-reactivity with TGF-β_3 with the TGF-β_1 antibody decreasing TGF-β_3 activity by approximately 20%. The TGF-β_2 antibody completely neutralised TGF-β_2 activity, although there was cross-reactivity with TGF-β_3, showing a reduction in activity of approximately 15%.

The CAT TGF-β_1 antibody becomes more potent *in vivo* due to unknown mechanisms, and this can be mimicked *in vitro* by the acid-activation of the antibody (personal communication from Dr Matthew McCourt, CAT). A titration of acidified TGF-β_1 antibody, supplied by CAT, was performed (figure 6.4A) to determine the optimal concentration for use *in vitro*. Increasing concentrations of antibody resulted in a dose-dependent decrease in TGF-β_1 activity, however this was also observed with the control antibody. At a concentration of 150 ng / ml the acidified TGF-β_1 antibody decreased TGF-β_1 activity by approximately 40% ($p<0.05$ compared with 0.1 ng / ml TGF-β_1 control group) whilst the control antibody was not significantly different from the control group (decrease of approximately 10%). Higher concentrations of antibody caused a further reduction in TGF-β_1 activity in both TGF-β_1 and control antibody treatments. Therefore, a concentration of 150 ng / ml was chosen to perform subsequent characterisation studies (figure 6.4B). At this concentration the acidified TGF-β_1 antibody decreased TGF-β_1 activity by approximately 35% compared to the control antibody, whilst having no neutralising effect on either TGF-β_2 or -β_3.

6.4 Determination of endogenous active and latent TGF-β isoforms produced in the conditioned media of normal mesothelial and AC29 cells in culture

Due to their efficiency and low cross-reactivity, the TGF-β_1 and -β_2 neutralising antibodies purchased from R & D Systems were used to quantitate the different TGF-β isoforms present in conditioned media collected from normal mesothelial and AC29 cell lines (figure 6.5). Conditioned media were heated to activate latent TGF-β present in the samples (section 2.6.1).

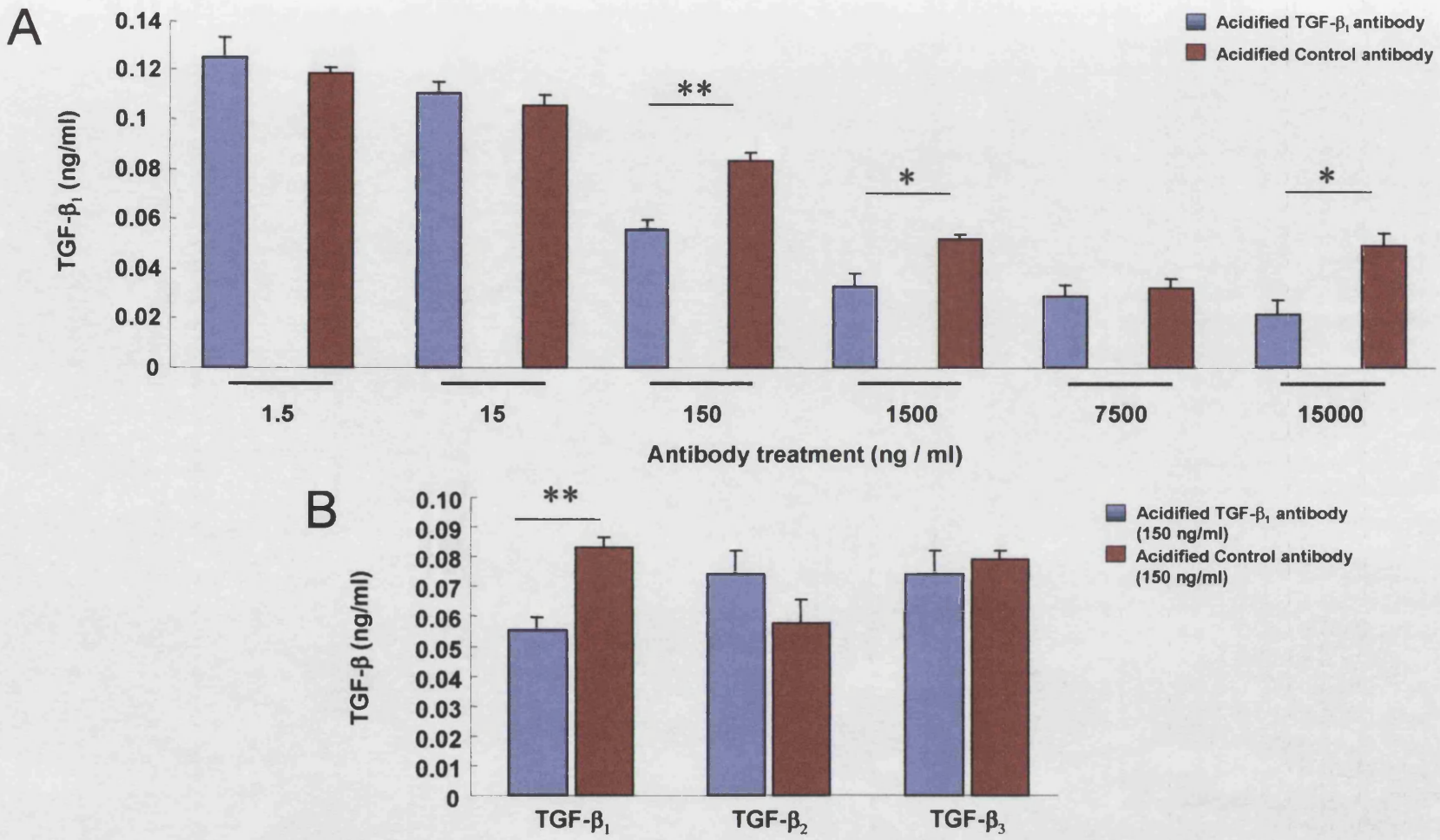


Figure 6.4 Titration and characterisation of CAT acidified TGF-β_1 antibody. **A** The efficacy of acidified TGF-β_1 antibody to neutralise 0.1 ng/ml TGF-β_1 was determined in a dose response titration. **B** Optimal concentrations of antibody were incubated with 0.1 ng/ml TGF-β_{1-3} for 30 min and the specificity and efficacy assessed via the MLEC TGF-β bioassay (section 2.6.2). Each bar represents the mean ± SEM for six replicates. *$p<0.05$, **$p<0.001$. Data are representative of two repeat experiments.

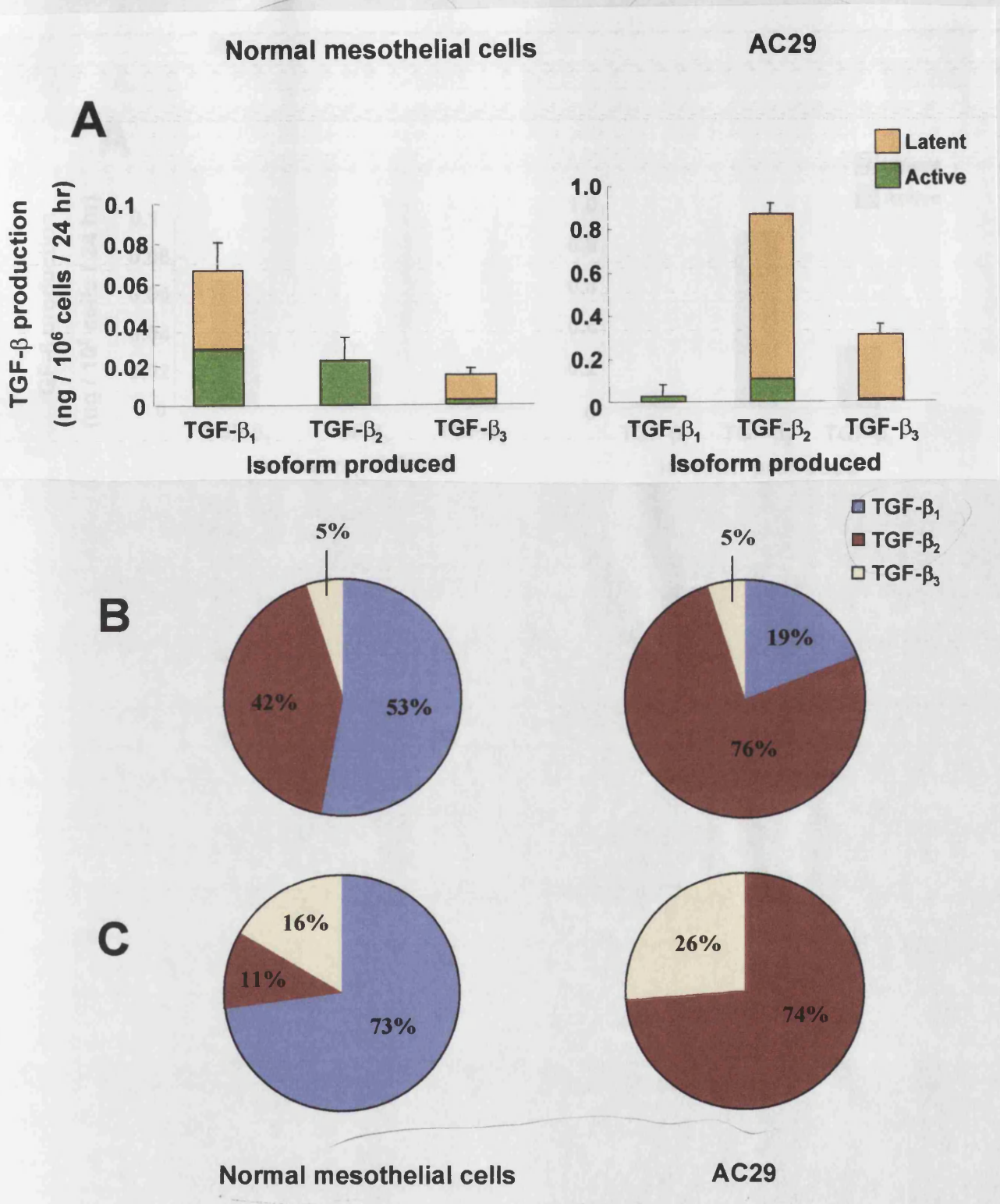


Figure 6.5 Quantification of active and latent TGF-β isoforms produced by normal and malignant murine mesothelial cells.. Conditioned media harvested from confluent normal mesothelial cells and malignant AC29 cells in culture were incubated with R & D TGF-β neutralising antibodies for 30 min and TGF-β activity assayed. Results were expressed as production of TGF-β_1, -β_2 and -β_3 (**A**) and as a proportion of active (**B**) and total (**C**) TGF-β. Results are representative of two repeat experiments.

As previously demonstrated in figure 3.3, AC29 cells produced elevated levels of TGF-β, the total amount over 12 times that of normal mesothelial cells (figure 6.5A). There was a shift in the proportion of TGF-β isoforms produced comparing the control cells to the malignant cells. Figure 6.5B demonstrates that the majority of active TGF-β produced by the normal control cells was the -β_1 isoform (53%) whereas the malignant cells produced predominantly TGF-β_2 (76%). This trend was also observed with total TGF-β production (figure 6.5C), the control cells produced TGF-β_1 as 73% of the total, whilst the malignant cells produced TGF-β_2 as 74% of the total.

6.5 The effect of neutralising specific endogenous TGF-β isoforms on AC29 cell proliferation and collagen production

Following the determination of the TGF-β isoforms produced by AC29 cells, the effect of inhibiting specific isoforms with neutralising antibodies on cell proliferation and collagen production was examined. Figure 6.6 demonstrates that R & D TGF-β antibodies had no effect on AC29 ^{3}H-TdR incorporation, with no significant changes observed between any of the treatment groups. The CAT TGF-β neutralising antibodies (figures 6.7A and 6.7C) and the acidified TGF-β_1 antibody (figure 6.7B) also had no effect on AC29 thymidine incorporation.

Basal AC29 procollagen production was assessed after incubation with R & D TGF-β neutralising antibodies (figure 6.8). Apart from the pan-specific TGF-β control antibody stimulating procollagen production, there were no significant differences between any of the treatment groups (pan-specific control antibody 9.90±0.11; pan-specific TGF-β neutralising antibody 8.28±0.24; medium control 6.98±0.60 nmol hyp / 10^6 cells / 24 hr, $p<0.05$ compared to TGF-β neutralising antibody and medium control). The effect of CAT TGF-β neutralising antibodies on AC29 procollagen production was also assessed (figure 6.9). There was no significant effect on the basal level of procollagen production with any of the treatments. However, there appeared to be a trend towards lowered procollagen production with TGF-β_2 neutralising antibody treatment.

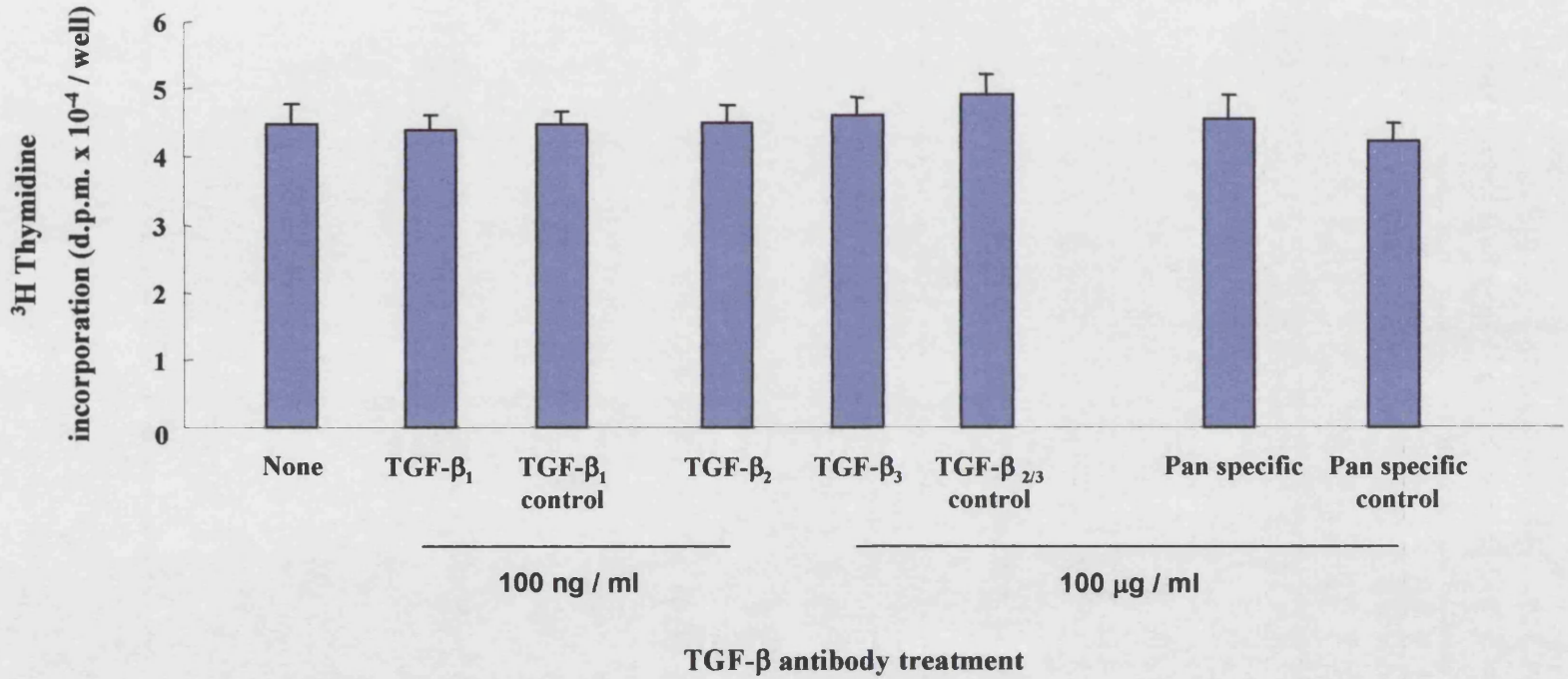


Figure 6.6 Neutralisation of specific and all TGF-β isoforms with R & D antibodies had no effect on AC29 DNA synthesis. AC29 DNA synthesis was assessed 24 hr after incubation with TGF-β neutralising antibodies. Results are expressed as disintegrations per minute (d.p.m.). Each bar represents the mean ± SEM for six replicate cultures. Similar data was obtained in six repeat experiments.

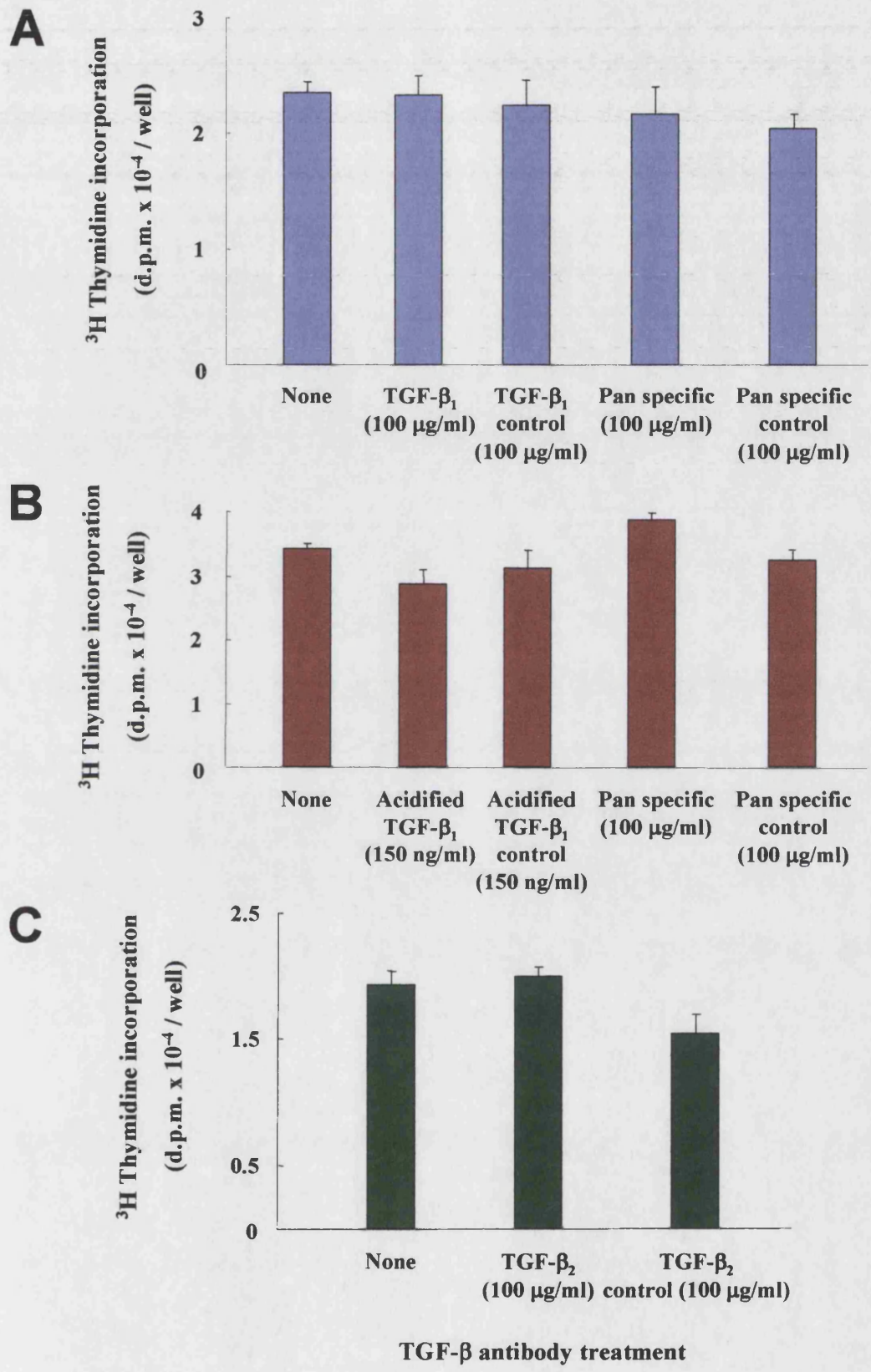


Figure 6.7 Cambridge Antibody Technology TGF-β antibodies did not affect AC29 DNA synthesis. AC29 DNA synthesis was assessed 24 hr after incubation with TGF-β neutralising antibodies. Results are expressed as disintegrations per minute (d.p.m.). Each bar represents the mean ± SEM for six replicate cultures. Similar data was obtained in three repeat experiments.

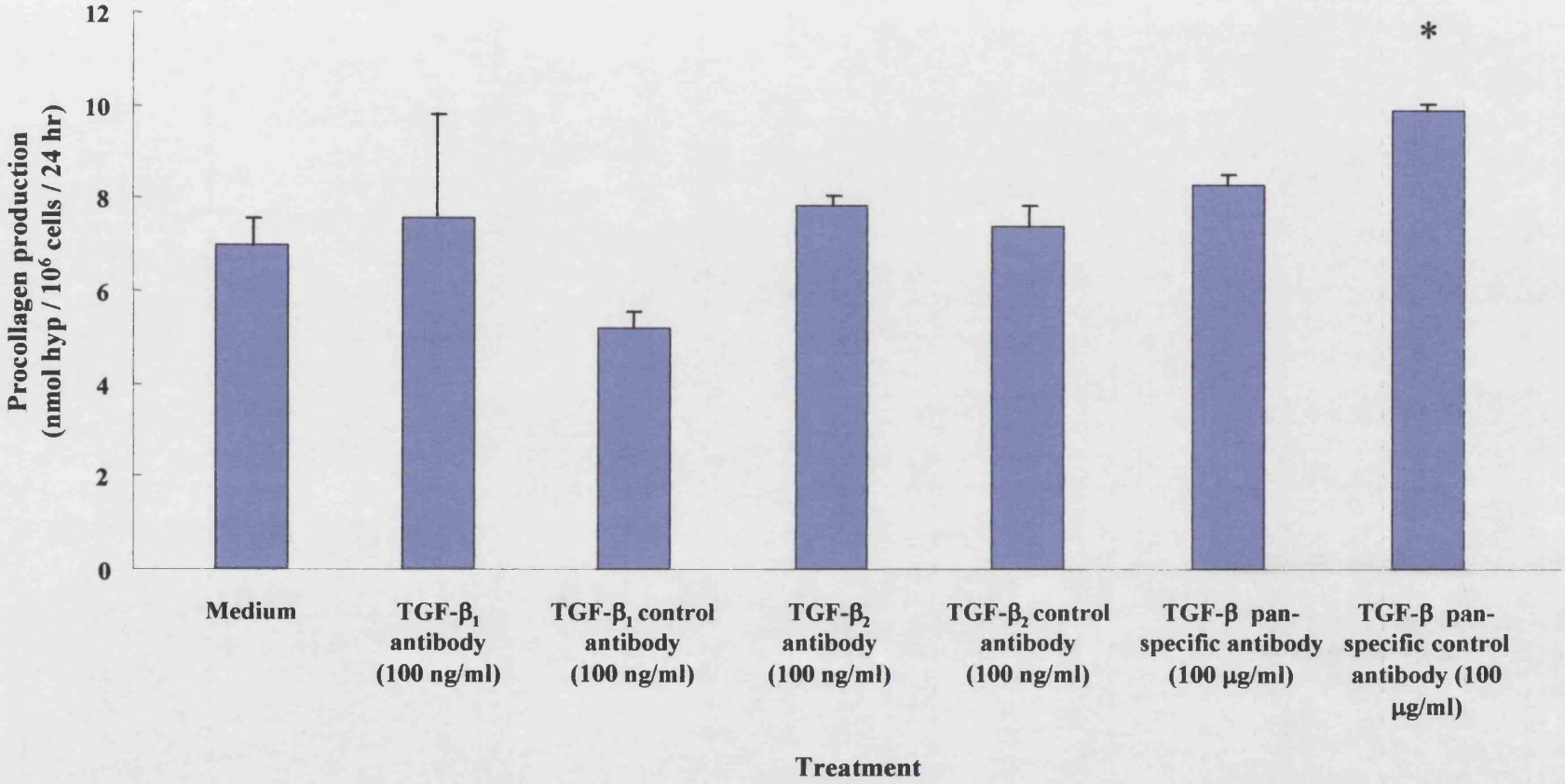


Figure 6.8 AC29 basal procollagen production was not affected by neutralising TGF-β with R & D antibodies. R & D TGF-β neutralising antibodies were applied to confluent cultures of AC29 cells for 24 hr. Values were corrected for cell number and procollagen production expressed as nmol hyp / 10^6 cells / 24 hr. Each bar represents the mean ± SEM for four replicate cultures. * denotes a significant difference of $p<0.05$ compared with both the medium control group and the pan-specific TGF-β antibody treated group. Data representative of two independent experiments.

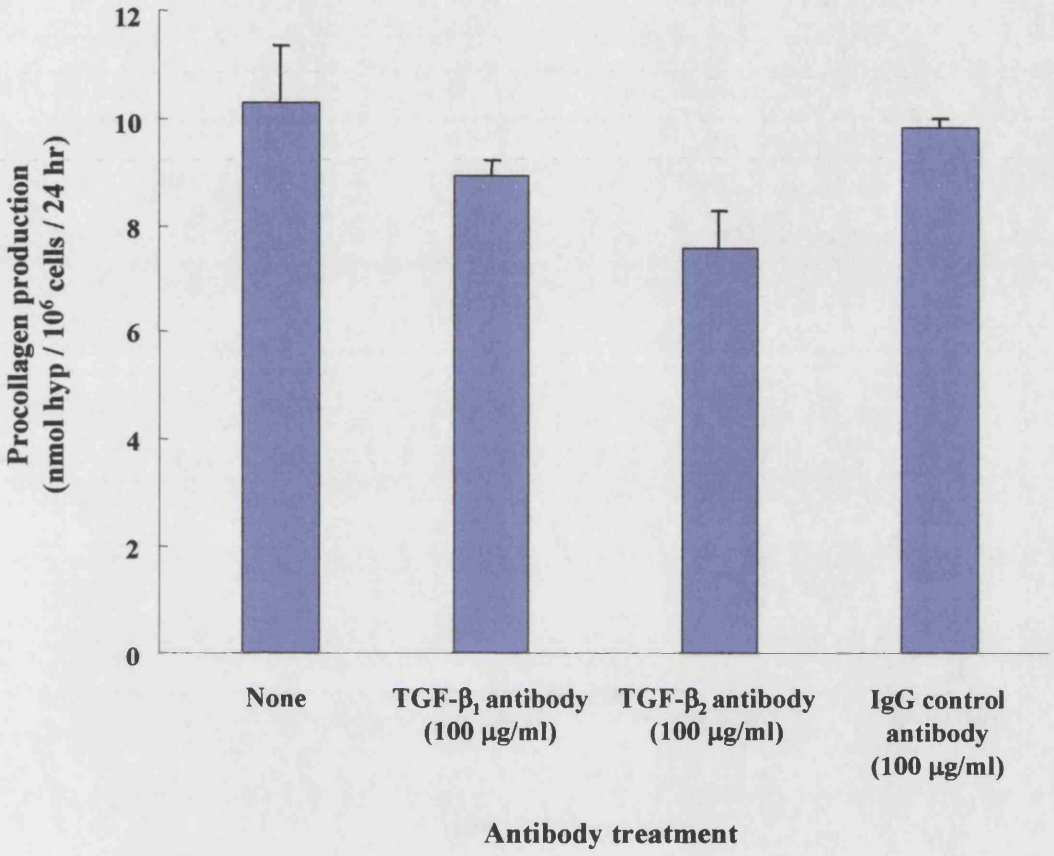


Figure 6.9 Neutralising TGF-β with CAT antibodies did not alter AC29 basal procollagen production. Confluent cultures of AC29 cells were incubated with CAT TGF-β neutralising antibodies with and without 1 ng/ml TGF-β_1 in DMEM supplemented with 1% FCS for 24 hr. Values were corrected for cell number and procollagen production expressed as nmol hyp / 10^6 cells / 24 hr. Each bar represents the mean ± SEM for six replicate cultures. Data representative of two independent experiments.

6.6 Discussion

6.6.1 Exogenous TGF-β_1, -β_2 and -β_3 inhibited mesothelioma cell proliferation

TGF-β is generally considered a tumour suppressor. In the early stages of tumourigenesis TGF-β inhibits cell proliferation, but as the disease progresses, mutations are gained in the TGF-β signalling pathway and TGF-β is no longer able to inhibit, in some cases stimulating tumour growth (section 1.3.3). To test the hypothesis that this switch has occurred in MM, exogenous TGF-β_{1-3} was applied to AC29 cells in an increasing concentration. Unexpectedly, proliferation was inhibited with all TGF-β isoforms in a dose responsive fashion (figure 6.1). All three isoforms of TGF-β had a virtually identical effect on inhibiting AC29 cell proliferation. Cordeiro *et al.* (2000) also observed no differences between the effect of different exogenous TGF-β isoforms *in vitro* on human ocular fibroblast proliferation, collagen gel contraction or cell migration.

Autocrine TGF-β levels are highly elevated in MM (figures 3.3 and 6.5) suggesting a role for TGF-β in MM biology. However, an *in vitro* study of MM showed that exogenous TGF-β_1 (0 – 10 ng/ml) had no effect on rat MM cells whilst stimulating normal mesothelial cells to proliferate (Kuwahara *et al.*, 2001). The rat MM cell lines produced 30 – 70 times more TGF-β than the control cells, and it was hypothesised by the authors that the high autocrine production of TGF-β had maximally stimulated the MM cell lines, and therefore further TGF-β application had no effect.

TGF-β is known to have biphasic effects on other cell types. For example, at low concentrations TGF-β stimulates fibroblast proliferation, but at higher concentrations inhibits proliferation (McAnulty *et al.*, 1997, Cordeiro *et al.*, 2000). It is possible that TGF-β has biphasic effects on AC29 cell proliferation, the high levels of endogenous TGF-β production are sufficient to stimulate proliferation, and application of further TGF-β increases the local concentration to levels resulting in inhibition.

TGF-β has been shown to reduce cell proliferation in other tumour settings whilst simultaneously enhancing other aspects of tumour growth. For example, TGF-β_1 inhibits the *in vitro* proliferation of human oral carcinoma cell lines, but also in some

of the same cell lines has a stimulatory effect on invasion (Hsu *et al.*, 2002). In another study of human oral squamous cell carcinoma it was demonstrated that TGF-β_1 increased cell migration whilst inhibiting cell proliferation (Hasina *et al.*, 1999). These studies provide evidence that TGF-β-induced cell migration and invasion may contribute to the tumour enhancing effects of TGF-β, and that cell proliferation may play a less important role. TGF-β has been shown to enhance MM tumour growth *in vivo* (Fitzpatrick *et al.*, 1994, Marzo *et al.*, 1997). The stimulatory effect of exogenous TGF-β on MM collagen production was shown in chapter 3 (table 3.1). Additionally, cell proliferation *in vitro* and tumour growth *in vivo* was stimulated by collagen (chapters 4 and 5). Therefore, as TGF-β promotes MM growth *in vivo*, the inhibitory effect of TGF-β on cell proliferation (figure 6.1) may be negated by the protumourigenic properties of TGF-β, such as enhanced collagen production, immune suppression and the stimulation of angiogenesis (section 1.3.2, figure 8.1).

Although exogenous TGF-β did not stimulate MM cell proliferation, it was hypothesised that inhibition of the elevated levels of endogenous TGF-β would reduce AC29 proliferation. In order to determine the relative contributions of specific TGF-β isoforms to AC29 cell proliferation and collagen production a panel of TGF-β-neutralising antibodies were characterised to determine their specificity and potency.

6.6.2 TGF-β neutralising antibody characterisation

TGF-β neutralising antibodies from two separate sources were characterised for their ability to neutralise porcine TGF-β_1 and -β_2 and recombinant human TGF-β_3. These isoforms of TGF-β were also used as the standards in the MLEC TGF-β bioassay to determine the different TGF-β isoforms produced in MM conditioned media.

When determining the ability of the TGF-β antibodies to bind and neutralise TGF-β isoforms, comparisons were made with the control antibodies rather than to a set 0.1 ng / ml concentration of TGF-β. TGF-β has an affinity for binding to plasticware used in tissue culture (Reisenbichler and Jirtle, 1994) and this could affect the retrieval of TGF-β and it's measurement. The 'sticky' nature of TGF-β probably accounts for the differences seen in the remaining TGF-β concentration of the same

control antibodies with different TGF-β isoforms (figures 6.2 – 6.4). Therefore, comparisons between control and neutralising antibodies were chosen as an indication of the potency of the antibody as both groups were treated in an identical fashion.

The R & D Systems TGF-β antibodies were highly potent, although the TGF-β_3 antibody cross-reacted with TGF-β_2, neutralising activity by 65% (figure 6.2). The cross-reactivity of the TGF-β_3 antibody made it unsuitable for characterising isoform production in conditioned media. However, the specificity of the other antibodies enabled the determination of TGF-β_1 and TGF-β_2 activity, and by the subtraction of these from the total, TGF-β_3 activity could also be assessed (figure 6.5).

The CAT TGF-β_1 neutralising antibody was not as potent as the R & D Systems antibody, with the CAT TGF-β_1 neutralising antibody there was a 25% neutralisation of TGF-β_1 activity, although a similar degree of neutralisation was observed with TGF-β_3 (figure 6.3). Acid-activation of the antibody increased specificity, the antibody only neutralising TGF-β_1 (figure 6.4B), and improved potency (35% TGF-β_1 neutralisation compared to control antibody). However, even with this degree of neutralisation, there is evidence suggesting that the CAT TGF-β_1 neutralising antibody has an effect *in vivo*. Administration of the same TGF-β_1 antibody into a murine model of asthmatic airway remodelling showed almost a complete block of the remodelling process with a significant reduction in the amount of subepithelial airway collagen deposition (Dr Alistair Reinhardt, personal communication). Additionally, other studies have demonstrated that TGF-β_1 is the predominant isoform upregulated in human fibrosis and in experimental fibrosis models (Coker *et al.*, 1997, 2001). The CAT pan-specific TGF-β antibody and TGF-β_1 neutralising antibody had a very similar effect in significantly reducing murine bleomycin-induced lung fibrosis (Carruthers *et al.*, 2002). Therefore, for the TGF-β_1 neutralising antibody to have a similar neutralising effect to the pan specific TGF-β antibody *in vivo*, activation of the antibody is likely to be occurring.

Although the R & D Systems antibodies were more potent, CAT generously supplied sufficient pan-specific, TGF-β_1 and -β_2 antibodies with relevant controls for *in vivo*

experiments and therefore animal studies were conducted with these antibodies (chapter 7). Both panels of antibodies were used for *in vitro* studies on cell proliferation and collagen production.

Once the antibodies were characterised, the relative amounts of TGF-β isoforms produced by normal and malignant cells at the protein level were assessed.

6.6.3 Endogenous TGF-β_2 production was increased in AC29

Figure 6.5 compared TGF-β isoform production at the protein level by MM and normal mesothelial cell lines. The malignant cells produced a higher overall concentration of TGF-β than the normal mesothelial cells (figure 6.5A), confirming the previous results observed in figure 3.3. There were differences in the relative proportion of TGF-β produced by malignant and normal cells. AC29 produced a higher proportion of TGF-β_2 compared to normal cells, which were producing mainly TGF-β_1. This suggested that elevated TGF-β_2 production may be characteristic of the malignant process, and that TGF-β_2 may be the TGF-β isoform most important in MM biology. Other studies support this concept; Marzo *et al.* (1997) demonstrated that antisense oligonucleotides (ODNs) to TGF-β_2 inhibited AC29 *in vitro* proliferation and reduced tumour growth in a flank model of MM. Fitzpatrick *et al.* (1994) used TGF-β_1 and TGF-β_2 ODNs to similar effect, although the ODNs cross-reacted with each other. The TGF-β_1 ODN inhibited TGF-β_2 and vice versa, therefore any inhibitory effects observed on tumour growth may well have been due to the inhibition of TGF-β_2 alone.

6.6.4 Specific inhibition of endogenous TGF-β isoforms did not alter cell proliferation or procollagen production

The effect of neutralising specific TGF-β isoforms on AC29 proliferation and collagen production was examined. The antibodies were used at concentrations in excess of those required to neutralise the TGF-β produced by the cells over the course of the experiments. The characterisation experiments used concentrations of antibody sufficient to neutralise 0.1 ng/ml of TGF-β (figures 6.2 – 6.4). Figure 6.5 shows that over 24 hr 10^6 AC29 cells produce 0.03 ng of active TGF-β_1, 0.11 ng of active TGF-

β_2 and 0.01 ng of active TGF-β_3 (a total of 0.15 ng active TGF-β for all isoforms). Cell proliferation assays were conducted at 4,000 cells / well which were left to adhere for 24 hr before applying the treatment conditions (section 2.2.1). AC29 has a cell doubling time of 27 hr (Davis *et al.*, 1992). Thus, by the time of treatment there would be approximately 8,000 cells, which would produce a total concentration of 8 pg/ml of all active isoforms of TGF-β. Therefore, at the concentration used, the antibodies were more than adequate to neutralise all the TGF-β produced by the cells. Considering the collagen assays, figure 3.4A shows that 6 x 10^5 cells in 1 ml were present at t_0. Therefore, the maximum active TGF-β production would be 0.06 ng/ml, again at a low enough concentration for the antibodies to fully neutralise all TGF-β activity.

Surprisingly, no changes in proliferation (figures 6.6 and 6.7) were observed. Fitzpatrick *et al.* (1994) demonstrated that the anchorage-dependent growth of MM cell lines (including AC29) was not affected by the ODN inhibition of TGF-β_2, although anchorage independent growth in soft agar and *in vivo* tumour growth were inhibited. A wider range of *in vitro* assays, such as growth in soft agar, would need to be performed to assess the effect of neutralising TGF-β isoforms on AC29 in tissue culture. As discussed in section 6.6.1, it is possible that no differences would be observed in proliferation, and that other tumour cell properties such as migration and invasion would be affected.

However, Marzo *et al.* (1997), observed a decrease in anchorage-dependent AC29 cell proliferation with ODNs to TGF-β_1 and -β_2, although the same group had not managed to obtain these results previously (Fitzpatrick *et al.*, 1994). Also, Kuwahara *et al.* (1997) obtained a decrease in both anchorage-dependent and –independent growth using ODNs to TGF-β_2 (although rat cell lines were used rather than murine). There is controversy over how representative of an *in vivo* tumour an *in vitro* cell monolayer is. Recent studies are using new techniques such as tumour cell spheroids to create cell-cell interactions in a 3D setting to model the *in vivo* environment more accurately (Guirado *et al.*, 2003). Differences observed in the studies cited above may well be due to the limitations of using a cell monolayer for proliferation studies.

Minor effects were observed on procollagen production (figures 6.8 and 6.9), although these were most likely non-specific effects as the control antibodies were also affecting procollagen production. Due to the lack of specificity, the effect of TGF-β_3 neutralising antibody on AC29 collagen production was not examined (figure 6.8). There is evidence in the literature to suggest that TGF-β_3 acts as an anti-scarring agent and prevents ECM formation (Shah *et al.*, 1995). With this observation it would be expected that TGF-β_3 neutralising antibody would increase collagen production. Although this was not investigated, exogenous TGF-β_3 had an identical effect to the other isoforms on proliferation (figure 6.1) and inhibition of TGF-β_3 also had a similar effect to neutralisation of other TGF-β isoforms (figure 6.6).

The lack of effect on cell proliferation and collagen production in response to the TGF-β antibodies was not due to incomplete neutralisation of TGF-β as sufficient concentrations of antibody were used. It is possible that endogenous TGF-β may not be important for AC29 cell proliferation or collagen production, and therefore TGF-β inhibition had no effect on either. However, exogenous TGF-β stimulated collagen production (figure 3.5), and collagen was shown to enhance cell proliferation (figures 4.1 and 4.9) and tumour growth (figures 5.5 and 5.11). Collectively, these data strongly suggest a role for TGF-β-induced collagen production in MM growth. Alternatively, the TGF-β antibodies may not have been able to neutralise the endogenous TGF-β before it bound to its receptor and generated a response. This hypothesis could be tested by blocking TGF-β production at the mRNA level, using specific ODNs to all three TGF-β isoforms. Inhibiting mRNA production would prevent any TGF-β protein being translated and the effect on cell proliferation and collagen production could be examined.

6.7 Summary and conclusions

At concentrations that promoted collagen production (figure 3.5), exogenous TGF-β decreased cell proliferation. However, endogenous levels of TGF-β were raised in the malignant cells, with increased TGF-β_2 protein production compared to normal cells. Inhibiting specific TGF-β isoforms had no effect on proliferation or collagen production.

Although unexpected results were obtained *in vitro*, TGF-β could possibly still enhance tumourigenesis *in vivo*. Tobin *et al.* (2002) over-expressed TGF-β_1 by stable transfection in human breast carcinoma and found seemingly contradictory results *in vitro* and *in vivo*. It was observed *in vitro* that TGF-β_1 over-expression lowered the rate of proliferation, although no alteration on invasion through a collagen type I coated membrane was seen. However, in an *in vivo* murine flank model, TGF-β_1 over-expression enhanced tumour growth and increased tumour invasion into local tissue and metastasis to distant organs. The authors suggest that the tumour enhancing effects of TGF-β_1 *in vivo* may be in part due to paracrine effects on other cells present in the tumour environment (figure 8.1). Also, TGF-β can enhance tumourigenesis *in vivo* through the stimulation of angiogenesis and suppression of the host immune response (section 1.3.2). In addition, TGF-β is a potent stimulator of collagen production and, as demonstrated in chapters 4 and 5, collagen stimulates MM cell proliferation and tumour growth. Therefore, although the results in this chapter demonstrated the inhibition of proliferation in culture with TGF-β, *in vivo* TGF-β may exert a stimulatory effect on MM tumour growth, possibly through an enhancement of collagen production. This was investigated in the final results chapter, by inhibition of TGF-β *in vivo* with the systemic administration of the neutralising antibodies characterised in this chapter to an animal model of MM.

Chapter Seven

Transforming Growth Factor-β antibody in vivo studies

7.1 Introduction

There is strong evidence to suggest a role for TGF-β in the promotion of tumourigenesis through stimulation of ECM production. Human melanoma cell lines transfected with TGF-β_1 adenoviral vectors induced more stroma in and around the tumour than control cells (Berking *et al.*, 2001). The TGF-β_1-induced increase in ECM (predominantly collagen and fibronectin) conferred a survival advantage protecting cells against apoptosis and enhancing melanoma growth *in vivo*. The occurrence and size of metastases were also increased. In another study, the stable transfection of human pancreatic carcinoma cells with TGF-β_1 led to the induction of desmoplasia *in vivo* by tumour derived TGF-β_1 which was not seen in control tumours (Lohr *et al.*, 2001). The authors suggested that tumour stroma may be beneficial for tumour growth. Based upon the observations above as well as the previous experiments in this thesis, the hypothesis for this chapter was constructed:

tumour-derived TGF-β induces ECM, which promotes *in vivo* tumour growth

This hypothesis was tested using the TGF-β neutralising antibodies characterised in the previous chapter in the animal model of MM described in chapter 5. The use of antibodies in cancer treatment is widespread, and monoclonal antibodies have been likened to "magic bullets", being able to specifically neutralise proteins involved in disease (Gura, 2002). The systemic administration of TGF-β antibodies to animal models is an established procedure, and a demonstrated reduction in ECM production has been observed. Pan-specific neutralising antibodies have been shown to reduce rat renal atrophy (Miyajima *et al.*, 2000), and the same TGF-β_2 antibodies used *in vivo* in this chapter (figures 7.3 and 7.4) have been shown to reduce diabetic rat kidney fibrosis (Hill *et al.*, 2001). In addition, antibodies to TGF-β_1 and -β_2 have been shown to reduce the lung collagen accumulation associated with murine bleomycin-induced fibrosis (Giri *et al.*, 1993). The importance of collagen production to MM growth was established in chapters 4 and 5, and this chapter investigated the effect of inhibiting the elevated levels of TGF-β in MM on tumour growth and tumour collagen production. More specifically to:

1. determine the effect of TGF-β antibodies on MM tumour growth *in vivo*,
2. establish whether histological differences between the treatment groups exist,
3. and to quantitate the collagen content of antibody treated tumours by HPLC.

7.2 The effect of TGF-β neutralising antibodies on tumour growth *in vivo*

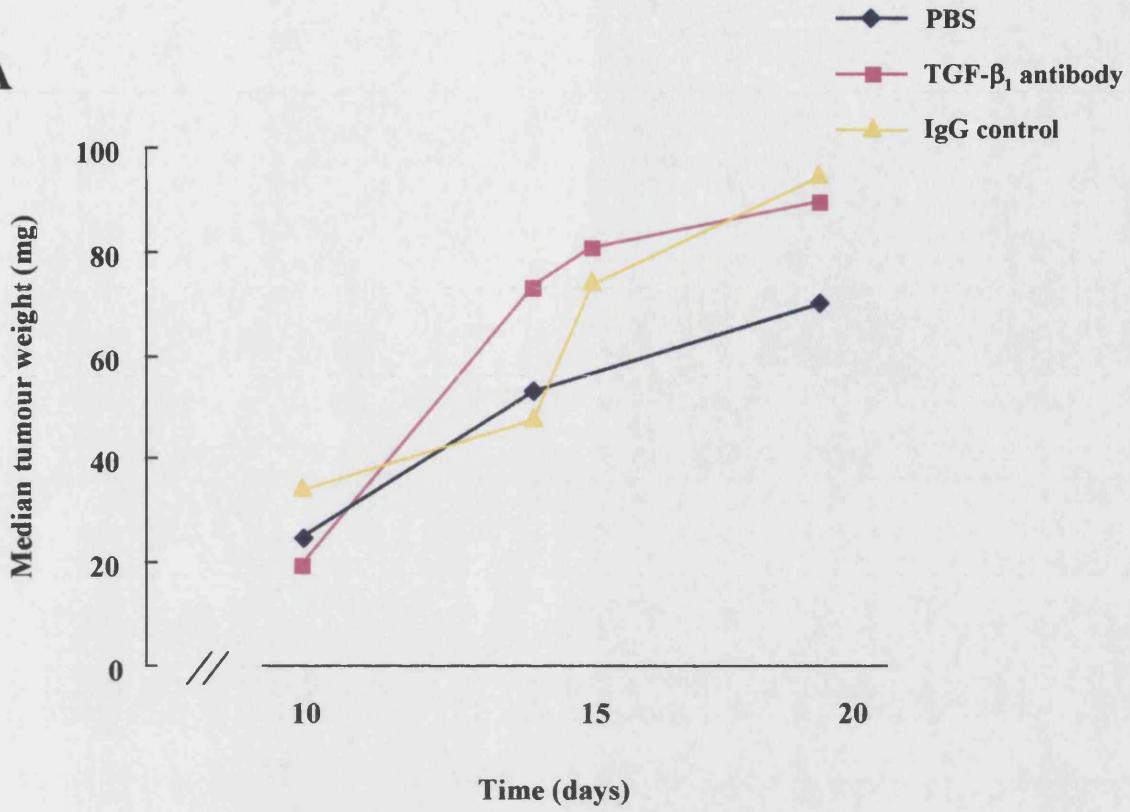
The *in vivo* relevance of different TGF-β isoforms on MM tumour growth was investigated by the i.p. administration of CAT TGF-β neutralising antibodies to the s.c. murine model of MM. The tumours were harvested at several time-points (10, 14, 15 and 19 days), the later time-points dependent upon tumour size and the ulceration of the subcutaneous tumours through the skin. Figure 7.1 shows the effect of TGF-β_1 neutralising antibodies on median tumour growth at 4 different time-points. Each group demonstrated a steady increase in median tumour weight over the course of the experiment, although considerable variation was observed within each group (figure 7.1**B**). A representative experiment demonstrating the spread of tumour weights at 14 days is shown in figure 7.2. There were no significant differences between any of the groups at any of the times examined.

Due to tumour ulceration through the skin, experiments with TGF-β_2 neutralising antibodies were unable to be continued beyond the 15 day time-point. However, administration of TGF-β_2 antibody significantly reduced median tumour weight at 15 days (figures 7.3 and 7.4). The TGF-β_2 antibody treated tumours exhibited more than a 50% reduction in median tumour weight compared with the PBS and IgG control groups, $p<0.001$ in both cases.

Pan specific neutralisation of all three TGF-β isoforms (figures 7.5 and 7.6) decreased median tumour weight compared to PBS control at 14 days (pan-specific TGF-β antibody 30 (14 – 66); PBS control 46.5 (25 – 109), $p<0.05$). However, at earlier and later time-points there were no differences between any of the treatment groups.

The weights of the animals were monitored at regular intervals over the course of the experiment, to ensure that antibody administration was not deleterious to animal health. Figure 7.7 shows the effect of antibody administration over 19 days. All of

A



B

Antibody treatment
Median tumour weight (mg)

Time (day)	*PBS control*	*TGF-β_1*	*IgG control*
10	24.5 (10-85)	19 (10-40)	34.5 (6-55)
14	53 (21-116)	72.5 (30-419)	48 (3-179)
15	-	80.5 (4-301)	76 (19-353)
19	70 (22-253)	89 (23-372)	95 (26-289)

Figure 7.1 The effect of TGF-β_1 neutralising antibodies on AC29 tumour growth. Mice were inoculated subcutaneously with 10^6 AC29 cells. Animals were injected intraperitoneally with 5 mg/kg (in 100 µl) of TGF-β_1 neutralising antibody, irrelevant IgG control antibody and 100µl of PBS thrice weekly. Groups of animals were sacrificed at 10, 14, 15 and 19 days and the induced tumours surgically excised. **A** compares the median tumour growth of the treatment groups over time, **B** demonstrates the median tumour weight (mg) with the range in parentheses.

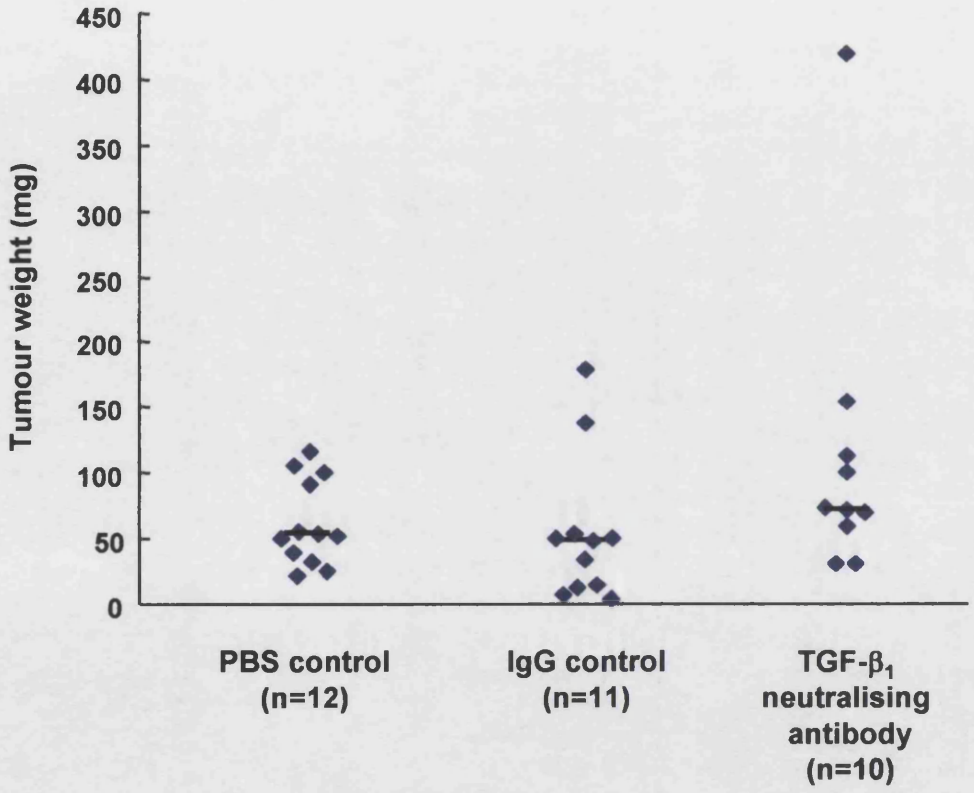
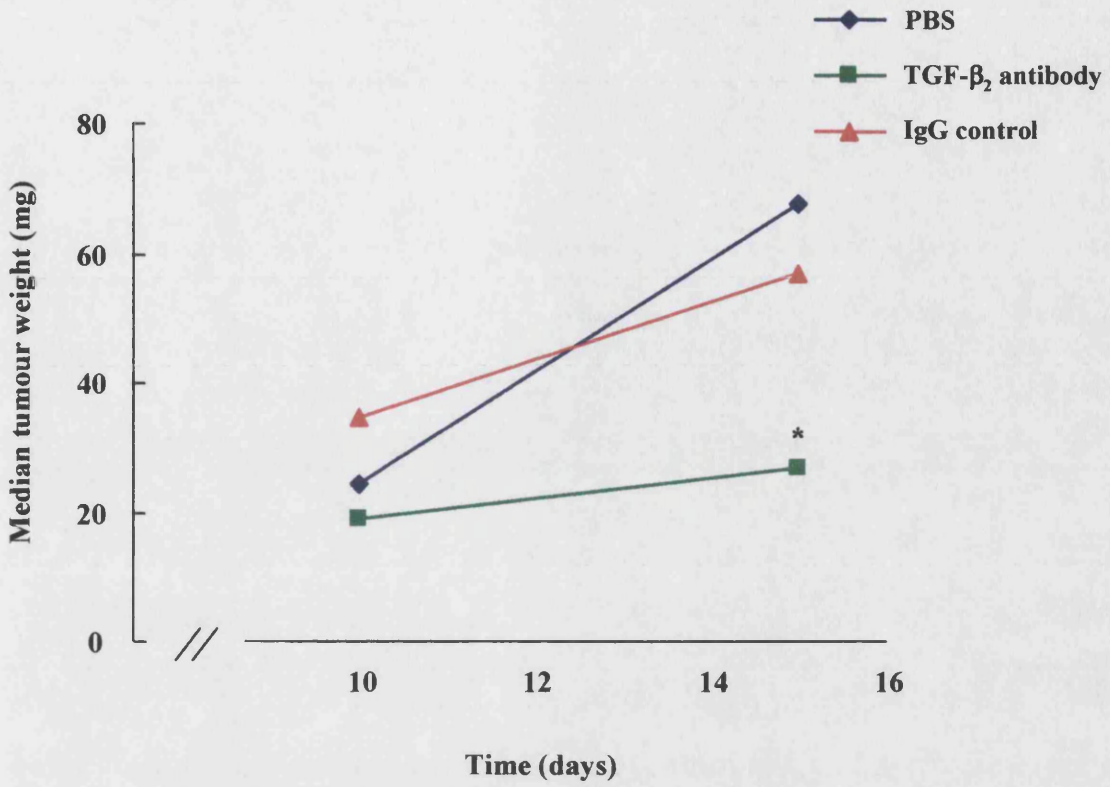


Figure 7.2 TGF-β_1 neutralising antibody did not affect tumour weight at 14 days. Female CBA mice were injected subcutaneously with 10^6 AC29 cells in 100 µl of DMEM. Animals were injected intraperitoneally with 5 mg/kg (in 100 µl) TGF-β_1 neutralising antibody, irrelevant IgG control antibody and 100µl of PBS thrice weekly. The animals were sacrificed after 14 days and the induced tumours surgically removed and weighed. The horizontal bar represents the median tumour weight. Results are representative of two repeat experiments.

A



B

Antibody treatment
Median tumour weight (mg)

Time (day)	*PBS control*	*TGF-β_2*	*IgG control*
10	24.5 (10-85)	19 (6-36)	34.5 (6-55)
15	67.5 (27-114)	27 (5-51) *	57 (35-193)

Figure 7.3 TGF-β_2 neutralising antibodies decreased AC29 tumour growth. TGF-β_2 neutralising antibody and irrelevant IgG control antibody (administered at 5 mg/kg in 100 µl) and 100µl of PBS were injected intraperitoneally into AC29 tumour bearing mice thrice weekly. Groups of animals were sacrificed at 10 and 15 days and the induced tumours surgically excised. **A** evaluates the median tumour growth of the treatment groups over time, **B** shows the median tumour weight (mg) with the range in parentheses. *$p<0.001$ compared to PBS and IgG control.

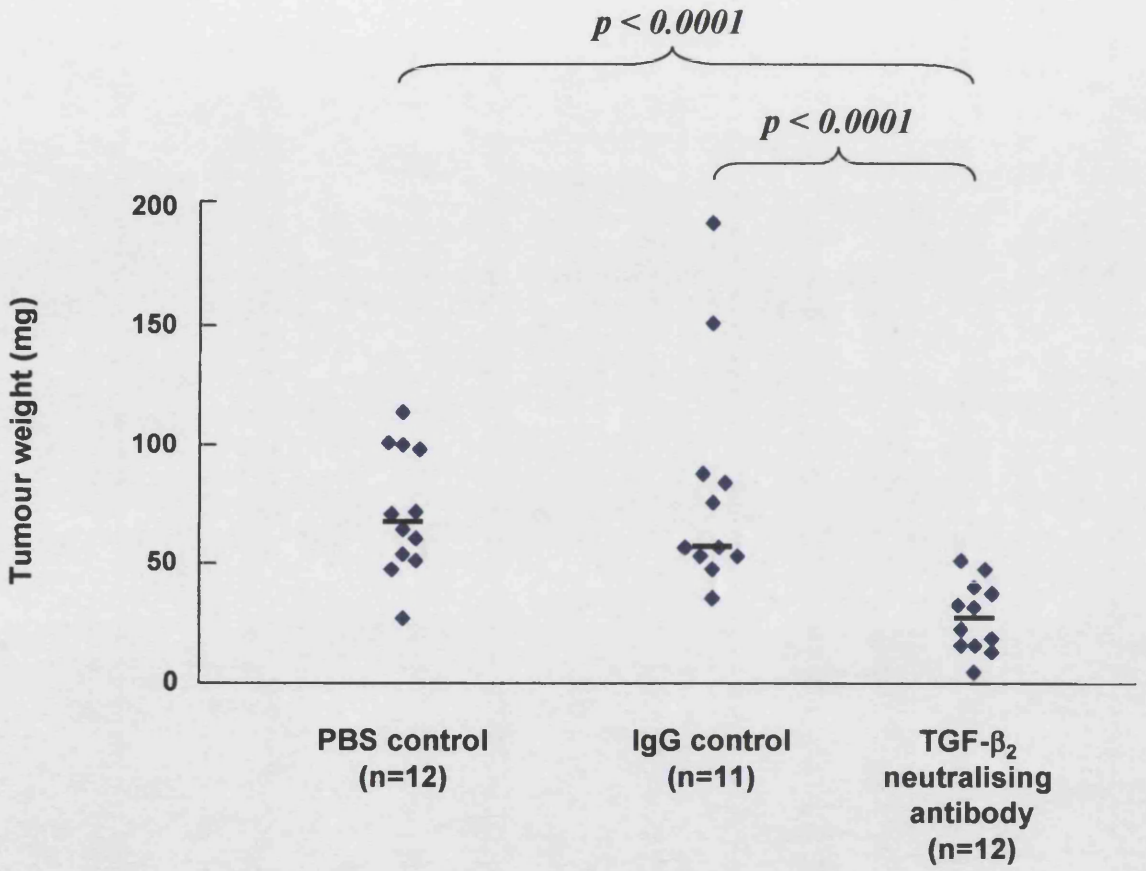
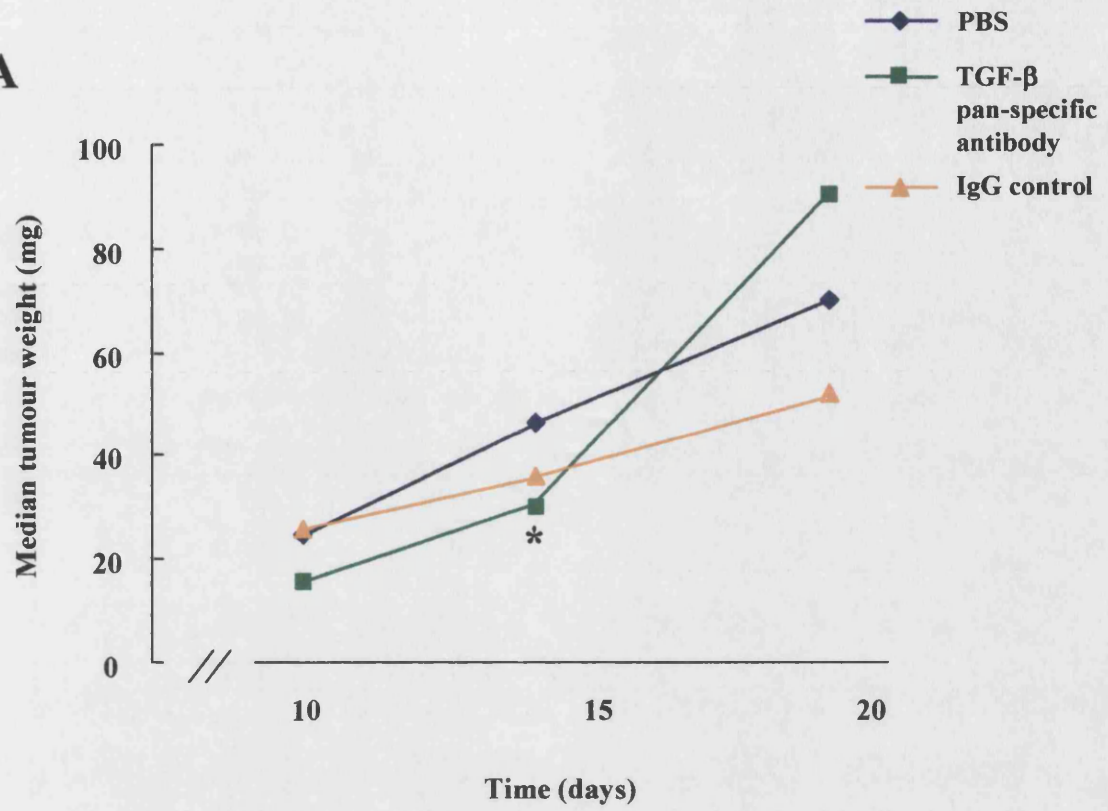


Figure 7.4 TGF-β_2 neutralising antibody decreased tumour weight at 15 days. Animals inoculated with AC29 tumour cells in the flank were injected intraperitoneally with 5 mg/kg (in 100 µl) TGF-β_2 neutralising antibody, irrelevant IgG control antibody and 100µl of PBS thrice weekly. The animals were sacrificed after 15 days and the induced tumours removed and weighed. The horizontal bar represents the median tumour weight.

A



B

Antibody treatment
Median tumour weight (mg)

Time (day)	*PBS control*	*Pan-specific*	*IgG control*
10	24.5 (10-85)	15.5 (4-76)	26 (5-79)
14	46.5 (25-109)	30 (14-66) *	36 (19-107)
19	70 (22-253)	90 (47-242)	52 (6-200)

Figure 7.5 The effect of pan-specific TGF-β neutralising antibodies on AC29 tumour growth. Mice seeded subcutaneously with 10^6 AC29 cells in each flank were injected intraperitoneally with 5 mg/kg (in 100 µl) of pan-specific TGF-β neutralising antibody, irrelevant IgG control antibody and 100µl of PBS thrice weekly. Groups of animals were sacrificed at 10, 14 and 19 days and the induced tumours surgically excised. **A** compares the median tumour growth of the treatment groups over time, **B** demonstrates the median tumour weight (mg) with the range in parentheses. *$p<0.05$ compared to PBS control

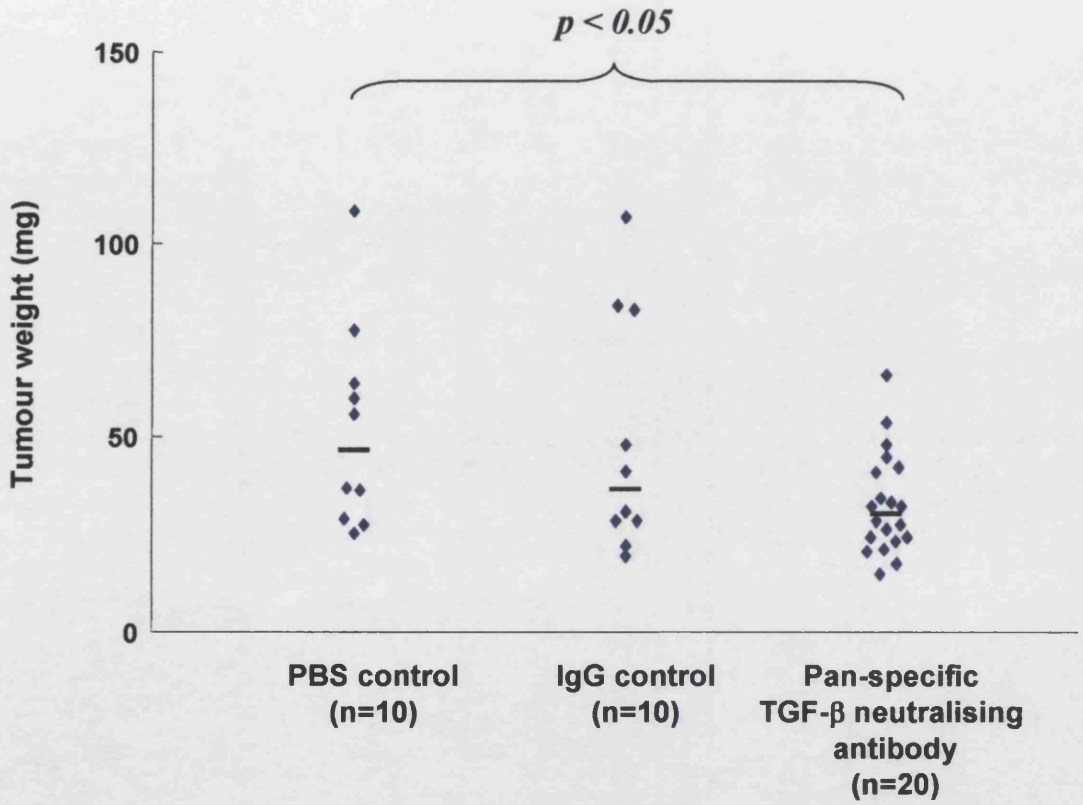


Figure 7.6 Pan-specific TGF-β neutralising antibodies reduced tumour weight at 14 days. Mice seeded with 10^6 AC29 cells in each flank were injected intraperitoneally with 5 mg/kg pan-specific TGF-β neutralising antibody, irrelevant IgG control antibody and PBS thrice weekly. The animals were sacrificed after 14 days and the induced tumours removed and weighed. The horizontal bar represents the median tumour weight.

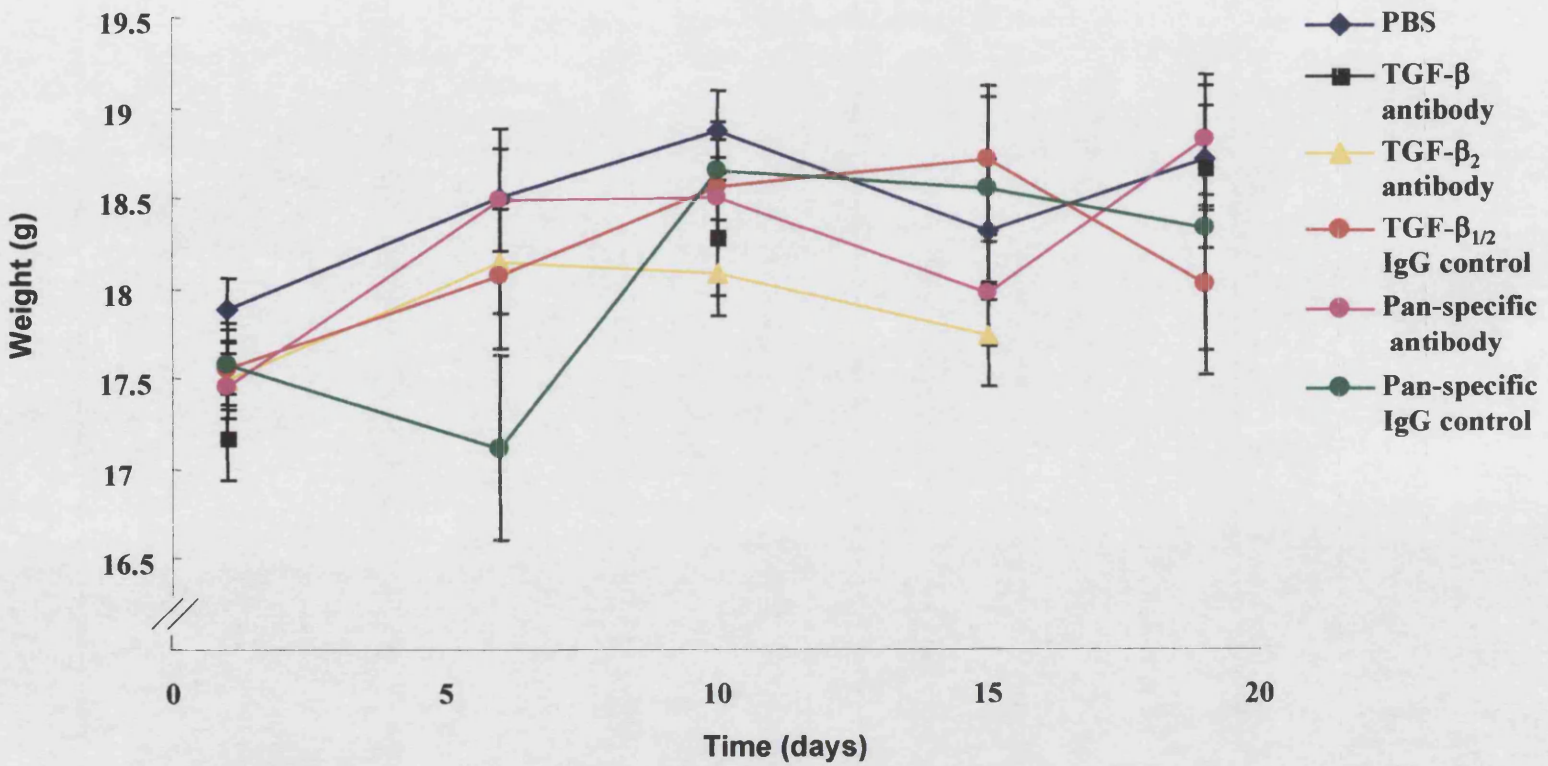


Figure 7.7 Administration of intraperitoneal TGF-β neutralising antibodies did not alter animal weights. The weight of the animals used in the antibody studies was monitored over the course of the experiments. The animals were weighed at regular intervals (4 - 5 days). Each point represents the mean weight of 5 animals ± SEM.

the groups had a similar increase in mean animal weight (approximately 5 – 10%) from the start of treatment.

7.3 Histology of tumour sections

Antibody treated and control tumours were processed to paraffin wax and sections cut for histological examination. To confirm the presence of TGF-β in the tumour samples, immunohistochemical staining with the R & D TGF-β_1 antibody was performed (figure 7.8). No positive staining resulted with the isotype control antibody. Dense TGF-β_1 staining was observed in the ECM of the tumour samples, equally distributed in the section. The different antibody treatments of the tumours resulted in no observable differences in TGF-β_1 distribution amongst the tumour groups.

To establish whether any differences in collagen distribution were evident histologically, the Martius scarlet blue stain for collagen was used. Figure 7.9 shows collagen staining for animals treated with TGF-β_1, TGF-β_2 and pan-specific neutralising antibodies (**A**, **B** and **C** respectively), with their control antibodies. Thick blue fibrils were observed within the tumours, indicative of a dense collagen network. There were no apparent differences between any of the antibody treatment groups and their controls. Also, as in the thiaproline studies (figures 5.17 and 5.18), inflammation was limited to the periphery of the tumour with virtually no infiltrate seen within the bulk of the tumour. From visual analysis the main inflammatory cells appeared to be macrophages.

7.4 Collagen analysis of tumour tissue

Tumour tissue from the 14/15 day *in vivo* experiments in this chapter was crushed and the collagen content assessed. This time-point was chosen as significant differences in tumour weight between treatment groups were observed at 14 - 15 days (figures 7.3 – 7.6). Collagen content was represented as total nM hyp / tumour and as a concentration per mg of tumour weight (nM hyp / mg tumour).

Treatment of animals with TGF-β_1 or TGF-β_2 antibodies did not alter the tumour collagen concentration (figure 7.10**A**). Correlation ($r = 0.47$) was found between

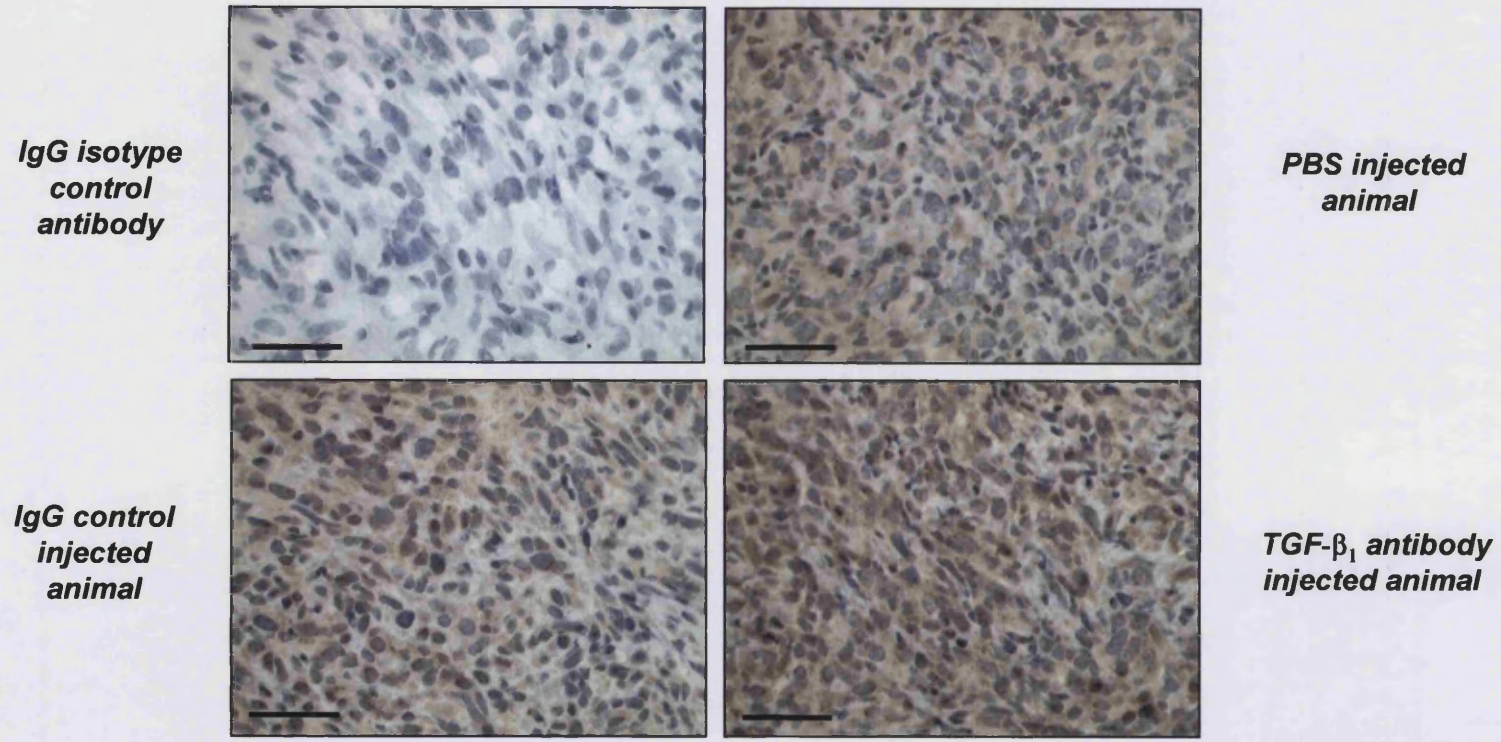


Figure 7.8 Immunohistochemistry for TGF-β_1 distribution in AC29 solid tumours. Female CBA mice were inoculated subcutaneously with 10^6 AC29 mesothelioma cells. Animals were injected intraperitoneally with 5 mg/kg (in 100 µl) TGF-β_1 neutralising antibody, irrelevant IgG control antibody and 100µl of PBS thrice weekly. The induced tumours were excised at 15 days, processed to paraffin wax and sections stained with a TGF-β_1 specific antibody. Bar represents 25 µm, original magnification x200.

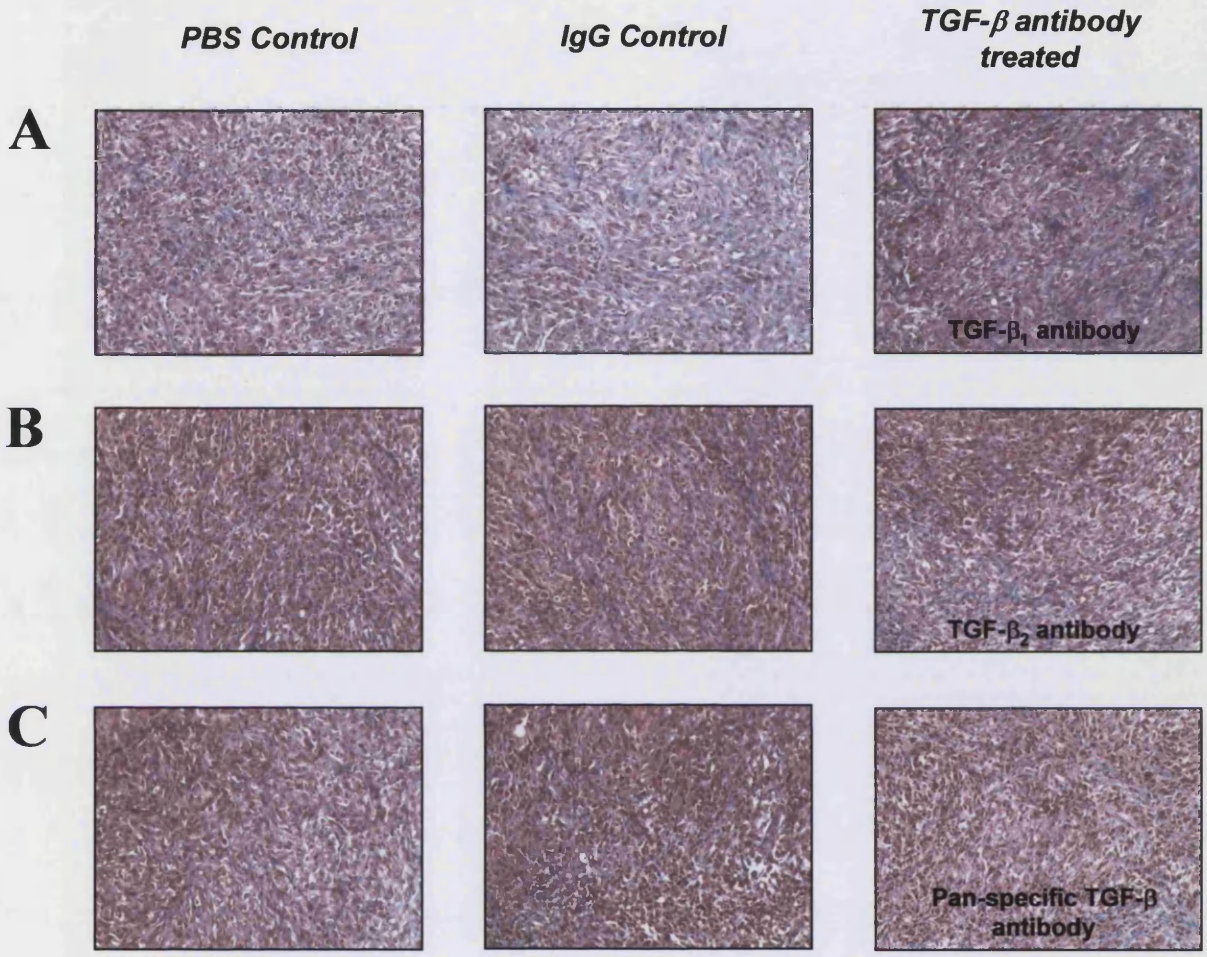


Figure 7.9 Martius scarlet blue staining revealed abundant collagen deposition in TGF-β antibody treated tumour sections. Female CBA mice were inoculated subcutaneously with 10^6 AC29 cells and groups treated with TGF-β_1 neutralising antibody (**A**), TGF-β_2 neutralising antibody (**B**), pan-specific TGF-β antibody (**C**), IgG control or PBS. The induced tumours were excised at 15 days, processed to paraffin wax and sections stained with Martius scarlet blue. Magnification x100.

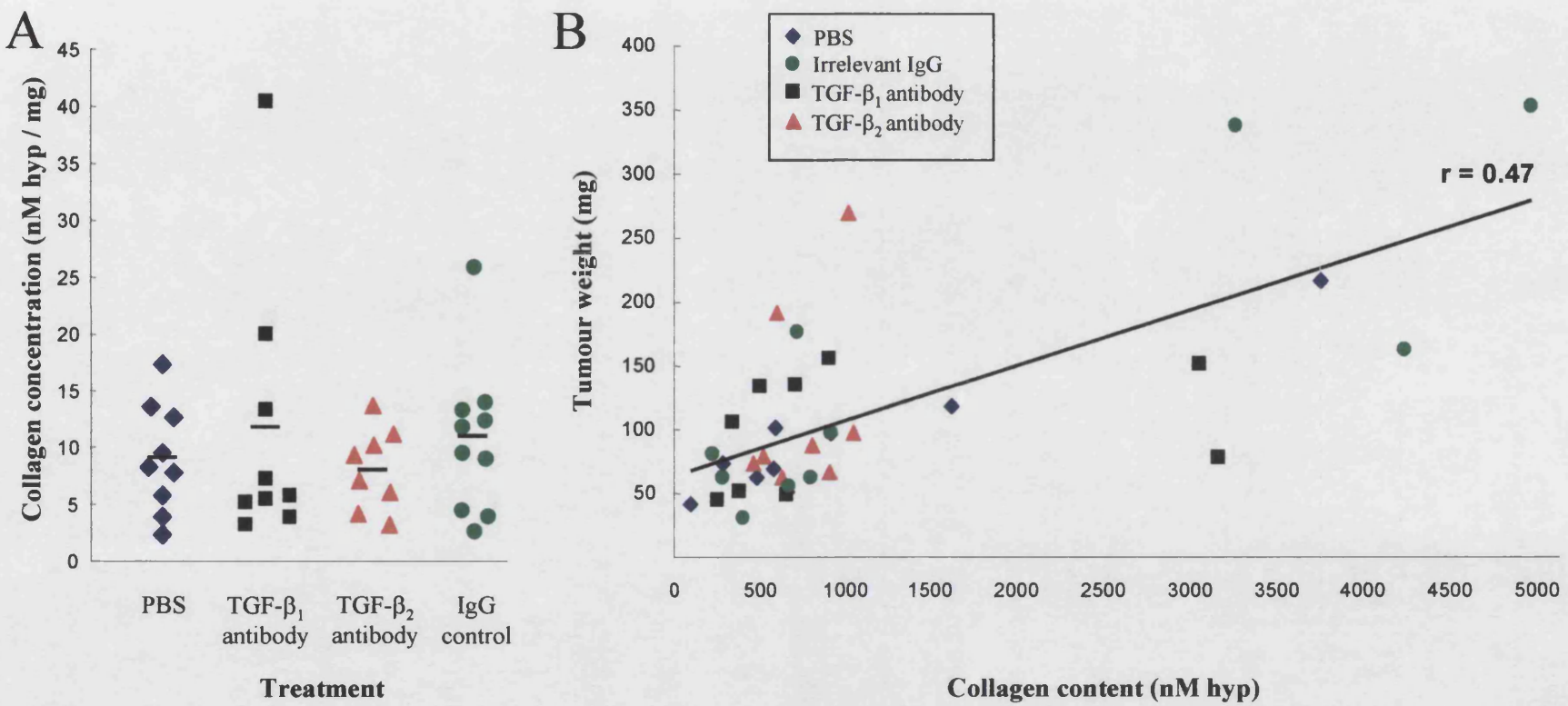


Figure 7.10 Administration of TGF-β_1 or TGF-β_2 neutralising antibodies had no effect on tumour collagen concentration at 15 days. Antibody treated and control tumours were analysed for hyp content using HPLC following excision at 15 days. The concentration of collagen per mg of tumour tissue was measured (**A**), the horizontal black bar indicative of mean collagen concentration. Also, tumour collagen content was plotted against tumour weight (**B**) and the correlation coefficient (**r**) calculated. Results are representative of two repeat experiments.

tumour weight and the total collagen content (figure 7.10**B**), an increase in tumour weight matched by an increase in total tumour collagen. Interestingly, the TGF-β_2 antibody treated group had a limited total collagen content, all tumours containing under 1000 nM hyp.

As shown in figure 7.11**A**, the neutralisation of all TGF-β isoforms with the pan-specific antibody reduced the concentration of collagen significantly compared to the PBS control group (pan-specific treated 4.03±0.68, PBS 9.11±0.76; $p<0.0001$). However, the IgG control antibody treated tumour group also demonstrated a lower collagen concentration compared to the PBS control group (control IgG treated 6.28±1.02; $p<0.05$ compared with PBS), although to a lesser extent. A strong correlation ($r = 0.78$) existed between tumour weight and collagen content (figure 7.11**B**), an increase in collagen related to an increase in tumour weight. The pan-specific antibody treated tumours were clustered in a group localised close to the y-axis, demonstrating that this group had a low total collagen content which was associated with a low tumour weight.

7.5 Discussion

7.5.1 In vivo administration of TGF-β neutralising antibodies

Tumour growth was not affected by TGF-β_1 neutralising antibody (figures 7.1 and 7.2). This could be due to the poor ability of the antibody to neutralise the substrate (figures 6.4 and 6.5). However, the same TGF-β_1 antibody reduced collagen accumulation in a murine model of asthmatic airway remodelling (Dr Alistair Reinhardt, personal communication), suggesting *in vivo* activity. Additionally, Carruthers *et al.* (2002) demonstrated an *in vivo* effect; the same TGF-β_1 antibody showed activity in a murine model reducing bleomycin-induced lung fibrosis. Therefore, the antibody is likely to be active *in vivo* and the lack of response seen is more likely due to the fact that TGF-β_1 neutralisation did not affect tumour growth. The lack of effect with TGF-β_1 neutralisation is consistent with the previous observation that the majority of active TGF-β produced by AC29 cells is TGF-β_2 (figure 6.5), and not TGF-β_1.

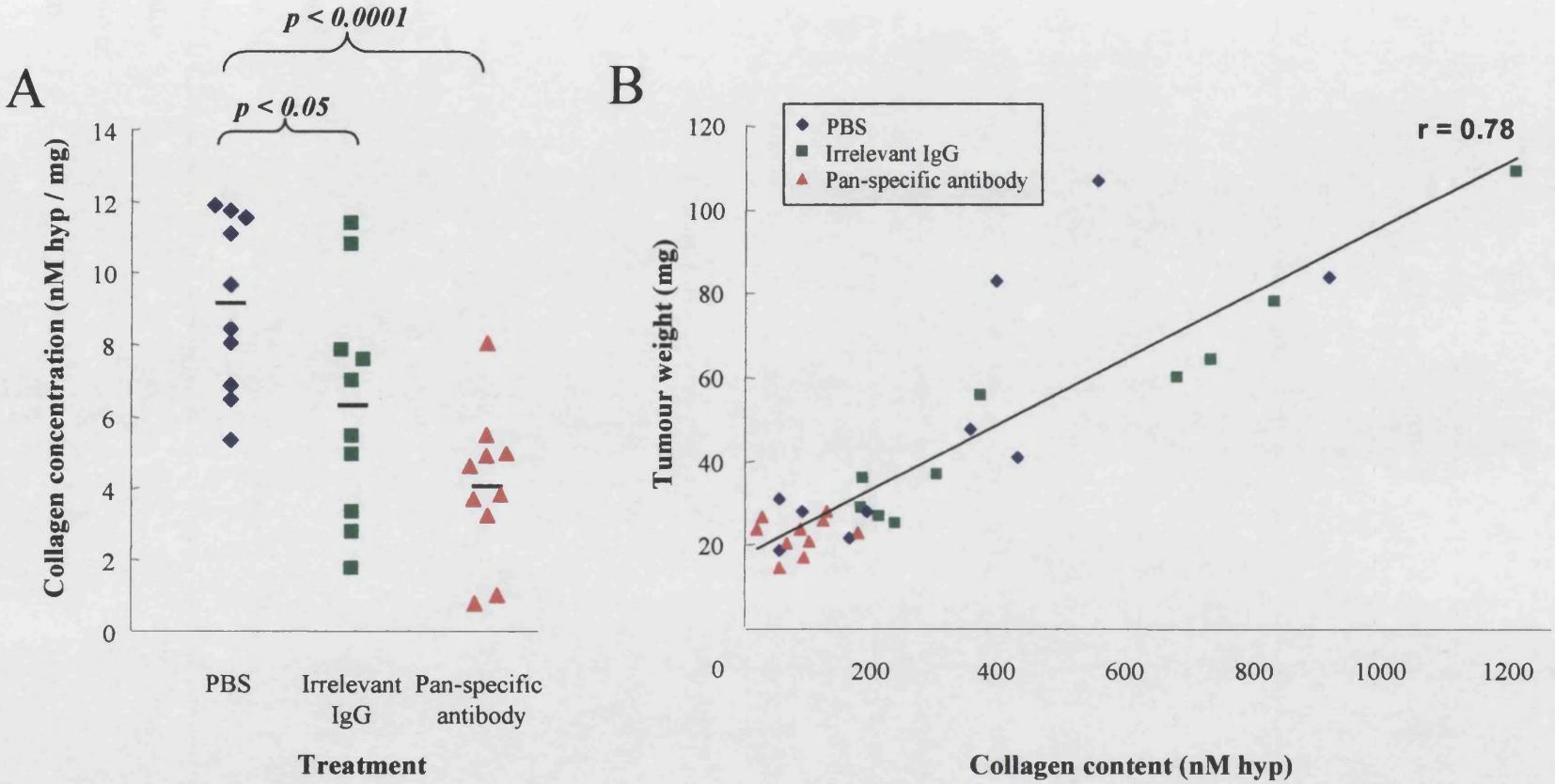


Figure 7.11 Administration of intraperitoneal pan-specific TGF-β neutralising antibodies reduced tumour collagen concentration at 14 days. Antibody treated and control tumours were analysed for hyp content using HPLC following excision at 14 days. The concentration of collagen per mg of tumour tissue was measured (**A**), the horizontal black bar indicative of mean collagen concentration. Also, tumour collagen content was plotted against tumour weight (**B**) and the correlation coefficient (**r**) calculated. Results are representative of three repeat experiments.

Fitzpatrick *et al.* (1994) observed decreased AC29 solid tumour growth with ODNs to TGF-β_1. However, the authors state that the ODN was not specific and also decreased TGF-β_2 mRNA levels. Therefore the effects observed may have been solely due to the neutralisation of TGF-β_2. Also, Shinozaki *et al.* (1997) observed that the injection of recombinant TGF-β isoforms into the subcutaneous tissue of newborn mice caused differing states of fibrosis depending on the isoform used. The level of fibrosis induced with TGF-β_1 was less when compared to injections of TGF-β_2 or TGF-β_3. This suggests that *in vivo* TGF-β_1 may be less important that the other TGF-β isoforms in matrix elaboration.

TGF-β_2 neutralising antibodies reduced tumour growth at 15 days compared to the PBS and IgG controls (figures 7.3 and 7.4). The experiments were unable to be taken to later time-points as the antibody treated tumours were extremely invasive through the skin and began to ulcerate by 15 days (figure 5.3A). Interestingly, TGF-β_2 was highly upregulated in MM compared to NM (figure 6.5) and inhibition of TGF-β_2 resulted in the decreased tumour growth observed, suggesting an important role for TGF-β_2 in MM biology. This supports previous studies (Fitzpatrick *et al.*, 1994, *Marzo et al.*, 1997) which also demonstrated a reduction in AC29 tumour growth by neutralisation of TGF-β_2. In another study examining eye development using TGF-β knockout mice, loss of expression of TGF-β_1 or TGF-β_3 had no effect on eye morphology whereas lack of TGF-β_2 expression resulted in decreased deposition of corneal stroma (Saika *et al.*, 2001). This observation is consistent with an important role for TGF-β_2 in ECM deposition.

However, TGF-β_2 antibody treated MM tumours did not contain a significantly lowered concentration of collagen (figure 7.10), and therefore it is likely that inhibition of TGF-β_2 reduced tumour size by other means. TGF-β promotes angiogenesis and host immune suppression (section 1.3.2), which are both crucial to successful tumour growth. Removal of TGF-β would possibly decrease angiogenesis and increase the inflammatory infiltrate into the tumour restricting growth. Specific staining for vascular and inflammatory markers on tumour tissue sections is required to confirm this. Additionally, the treatment of AC29 tumours with ODNs to TGF-β_2

resulted in reduced growth, with an increase in T cell lymphocyte infiltration into the tumours (Fitzpatrick *et al.*, 1994). This suggests that the mechanism of decreased tumour growth mediated by TGF-β_2 may be due to an increase in inflammatory cells, rather than the attenuation of collagen production.

Neutralisation of all TGF-β isoforms reduced tumour weight at 14 days compared with the PBS control (figures 7.5 and 7.6). Although tumour weight was not significantly reduced with pan-specific TGF-β neutralisation when compared with the IgG control treatment, there was a trend towards decreased tumour weight (figure 7.6). This result corresponds with the effect of specifically neutralising TGF-β_2. The effect of neutralising TGF-β_3 alone could not be examined as the antibody was not specific, showing 65% cross-reactivity with TGF-β_2 (figure 6.2). AC29 cells produce more TGF-β_3 than TGF-β_1 (figure 6.5) which suggests a possible role for TGF-β_3 in the tumourigenicity of MM. However, the neutralisation of all TGF-β isoforms with the pan-specific antibody did not have a more pronounced effect than specific neutralisation of TGF-β_2 alone, and therefore it could not be concluded that TGF-β_3 significantly influenced MM growth.

Reduced tumour growth was observed with the TGF-β_2 and pan-specific neutralising antibodies at the middle time-point of 14 – 15 days, which was not seen at 10 days (figures 7.4 and 7.6). However, with all the antibody treatments at 10 days the median value for the antibody group was lower than the PBS or IgG control groups, although not significant, perhaps due to insufficient power to detect the differences. This may be due to the difficulties in dissecting out small tumours from the surrounding tissue at the early time-point leading to erroneous weight measurements. An increased number of animals per treatment group may reveal significant differences at the 10 day time-point. Alternatively, the delay before the effect of the antibodies became apparent may have been because sufficient time was required to increase the circulating concentration of TGF-β antibody to a level adequate for tumour TGF-β neutralisation.

In the pan-specific antibody treated tumours, the reduction in growth was lost by 19 days (figure 7.5), with no difference apparent between the treated and untreated

tumours. Antibodies raised in one animal and then introduced into another animal will be recognised as foreign by the recipients' immune system and illicit an immune response, even if from the same species (Kunkel *et al.,* 1963). It is likely that in response to the introduced TGF-β neutralising antibodies the mice produced anti-idiotype antibodies. With repeated dosing, high affinity neutralising antibodies become apparent to the immune system between 14 – 21 days (Dr Ken Smith, personal communication). Therefore, in this study, by 19 days the introduced antibodies may have been neutralised by anti-idiotype antibodies and no longer had an effect. In addition to this, it is also possible that by the later time point a sufficient amount of time had elapsed to allow ECM deposition to occur, especially collagen production, further enhancing tumour growth (as demonstrated in figures 4.1 and 4.9). It is interesting to note that in another study using the same TGF-β_2 antibody delivered systemically *in vivo,* a time-point of 14 days was chosen to observe the results (Hill *et al.,* 2001). This suggests that the most potent effects of the antibodies may be elicited during the first two weeks of administration.

The same dose of antibodies used in these studies has been used in other *in vivo* experiments without detrimental effects to the animals (Cordeiro *et al.,* 1999, Hill *et al.,* 2001, Dr Alistair Reinhardt, personal communication). Also, in a murine breast cancer model, transgenic mice expressing a TGF-β antagonist over their entire lifetime had decreased levels of TGF-β and associated metastases without the regulatory roles of TGF-β in normal tissues being affected (Yang *et al.,* 2002). In this study animal weight was measured over the course of antibody administration (figure 7.7) and the unaltered animal weight observed was taken to show that the antibodies were not affecting animal health.

7.5.2 Histological and biochemical analyses of 14 – 15 day solid tumours

It was demonstrated histologically that TGF-β_1 was present in solid AC29 tumours (figure 7.8). The TGF-β antibodies administered *in vivo* neutralised activity by binding to the active site of TGF-β, thus preventing TGF-β binding to its receptor. The same antibody was used for TGF-β_1 immunohistochemistry on tumour tissue sections, and therefore any TGF-β inactivated by the *in vivo* antibody treatment should not be detected by histology due to a blocked active site. It was predicted that

the antibody treated tumour sections would stain less intensely for the presence of TGF-β_1. However, no differences were seen between the different antibody treatment groups. This could be due to the fact that a 2-dimensional tissue section may not be representative of an entire 3-dimensional structure. Further analysis of the whole tumour tissue may have revealed differences between treated and untreated tumour TGF-β content. For example crushing and homogenising entire tumours and assaying aliquots for TGF-β activity using the MLEC assay may have given a more accurate measure of active TGF-β content. However, all of the frozen tumour specimens obtained were used for collagen analysis. Staining for TGF-β_2, the predominant isoform produced by AC29 cells (figure 6.5), and TGF-β_3 was not possible, as suitable antibodies were unavailable.

In addition, no differences were observed histologically for collagen deposition between antibody treatment groups (figure 7.9), perhaps for the same reason as given above. Also, in agreement with the histological assessment, HPLC analysis revealed no differences in the collagen content of TGF-β_1 antibody and TGF-β_2 antibody-treated tumours (figure 7.10). As the TGF-β_2 antibody-treated tumours were significantly growth inhibited compared to the controls (figure 7.4) another mechanism unrelated to decreased collagen deposition was required to explain this reduction. Previous studies have also demonstrated that inhibition of TGF-β_2 in AC29 solid tumours reduced tumour growth (Fitzpatrick *et al.*, 1994, Marzo *et al.*, 1997). In these studies an increase in tumour infiltrating lymphocytes into the tumour mass was observed, suggesting that removal of the inhibitory effect of TGF-β on the host immune system was responsible for decreased tumourigenicity. However, the authors did not examine the effect of inhibiting TGF-β_2 on tumour stroma formation. In the current study, no gross differences in the inflammatory infiltrate were observed histologically in the tissue sections (figure 7.9). Further studies with markers specific to inflammatory cells are needed to investigate this in more detail. Interestingly, the TGF-β_2 antibody treated tumours had the lowest collagen content (figure 7.10A) compared to the other treatment regimes, and as a group had the lowest total collagen content / tumour size. Although not significant, there was a trend towards a lowered collagen content in the TGF-β_2 antibody treated tumours. Also, a trend towards reduced collagen production with TGF-β_2 antibody treated MM cells was observed *in*

vitro (figure 6.9). These data suggest that TGF-β_2 may participate in collagen-driven MM tumour growth to a certain extent.

Pan-specific neutralisation of all TGF-β isoforms led to an associated decrease in collagen and a decrease in tumour weight (figure 7.11). This is consistent with the results observed in chapters 4 and 5, showing that inhibition of collagen production reduced tumour growth and that smaller tumours contained a lower concentration of collagen. The concentration of collagen (figure 7.11**A**) followed the same trend as tumour weight (figure 7.6) suggesting a relationship between collagen and tumour growth, confirming the thiaproline *in vivo* studies (figure 5.5). Also, a strong correlation was found between tumour weight and total collagen content (figure 7.11**B**), providing further evidence for the importance of collagen to MM tumour growth. It is interesting to note that pan-specific neutralisation of all TGF-β isoforms had a more profound effect on tumour collagen content than inhibition of TGF-β_2 alone. Also, inhibition of TGF-β_1 had no effect on tumour collagen content (figure 7.10), therefore the effect of the pan-specific antibody may be due to inhibition of TGF-β_3. To test this hypothesis, the effect of exogenous application of the different TGF-β isoforms on MM collagen production needs to be investigated *in vitro.*

7.5.3 Non-ECM related effects of TGF-β

As well as the induction of ECM production, which stimulates tumour growth (chapters 4 and 5), TGF-β has many other pro-tumourigenic properties, and it is likely that all of these effects working in concert contribute towards increased tumour growth (see figure 8.1). As mentioned above, TGF-β is a powerful suppressor of the immune system, and inhibition of TGF-β_2 in MM has been demonstrated to increase the inflammatory infiltrate into tumours resulting in reduced tumour growth (Fitzpatrick *et al.,* 1994, Marzo *et al.,* 1997). However, evidence also exists supporting a role for collagen in reduced inflammatory infiltrate, as tumour stroma may form a barrier limiting inflammatory influx. Histological assessment of approximately 100 different cervical cancer sections revealed a significant correlation between TGF-β_1 production and the extent of tumour stroma, especially collagen type IV deposition (Hazelbag *et al.,* 2002). In addition, TGF-β_1 expression and collagen deposition were inversely correlated with the extent of the inflammatory infiltrate,

indicating that high levels of collagen and TGF-β have an inhibitory effect on inflammatory cell invasion. However, in the current study antibodies to TGF-β_2 had the greatest effect on tumour growth (figure 7.4) but had little effect on tumour collagen concentration (figure 7.10). It is likely that the reduced tumour size that was observed with TGF-β_2 neutralising antibodies may have been due to increased inflammation.

TGF-β is also a well-characterised stimulator of angiogenesis. For example, in a murine breast carcinoma model TGF-β_1 was demonstrated to increase blood vessel formation by the induction of VEGF (Breier *et al.*, 2002). Inhibition of TGF-β in other tumours has been shown to decrease the extent of vascularisation and reduce tumour growth. Incubation of a human model of prostate cancer with TGF-β_1 antibody or LAP resulted in smaller tumours with reduced surface vasculature (Tuxhorn *et al.*, 2002b). Additionally, histology revealed fewer blood vessels within the tumour mass compared to control tumours. The authors of this study suggested that a combination of TGF-β and stroma is required for tumourigenesis, with TGF-β acting in a paracrine manner on stromal cells to promote angiogenesis and tumour growth. Furthermore, in a study on colorectal cancer there was a significant correlation between TGF-β_1 and vessel density, depth of invasion, and the stage of disease (Xiong *et al.*, 2002). Also, in non-small cell lung cancer, tumour tissue TGF-β_1 levels correlated with vascular density and disease stage (Hasegawa *et al.*, 2001), and interestingly those patients with an elevated level of intratumoural TGF-β_1 had a trend towards decreased survival.

In the majority of tumours high TGF-β levels have been demonstrated to induce apoptosis. However, in a human breast cancer cell line inhibition of TGF-β signalling by expression of a soluble TGF-β type III receptor increased the level of apoptosis *in vitro* and *in vivo* (Lei *et al.*, 2002). This demonstrated that autocrine TGF-β production prevented apoptosis and promoted tumour growth in this model. Elevated levels of TGF-β are also observed in MM (figures 3.3 and 6.5), whilst no obvious areas of necrosis were observed within the tumour sections. It is possible that the

high levels of TGF-β found in MM may be preventing apoptosis, although further studies with markers specific to apoptotic cells are required to confirm this.

In addition to autocrine effects, tumour derived TGF-β has paracrine effects on stromal cells with the tumour ECM, leading to enhanced tumour growth, invasion and metastasis (Berking *et al.*, 2001, Tobin *et al.*, 2002). TGF-β produced by human prostate carcinoma tumour cells can activate stromal fibroblasts to the myofibroblast phenotype resulting in an increase in collagen type I production (Tuxhorn *et al.*, 2002a). Also, TGF-β_1 induces the transformation of human mesothelial cells to a myofibroblastic phenotype with an increase in the amount of collagen type I synthesised (Yang *et al.*, 2003). An increase in collagen type I production has a potentially stimulatory effect on MM cell proliferation (figure 4.1). All isoforms of TGF-β are chemotactic for fibroblasts and strongly upregulate fibroblast procollagen production (Coker *et al.*, 1997, Cordeiro *et al.*, 2000). No quantitation of stromal cells within the solid tumours was performed in this thesis, therefore the extent of stromal cell involvement in MM tumourigenesis is unknown. However, around the periphery of the tumours there was an area of intense matrix deposition creating a capsule keeping the tumour discrete from the host tissue. The tumour capsule contained cells at the interface between tumour and host that appeared to be fibroblasts, raising the possibility that stromal cells could be involved in this response.

The histological assessment carried out on the tumour sections revealed no obvious difference between inflammatory influx, vascularisation, apoptotic bodies or any differences in stromal cells between the different treatment groups (figure 7.9). Further histological investigation is required with specific markers for each area of interest to elucidate their contribution to tumour growth in more depth. However, despite these other possible effects of TGF-β, there was a decreased concentration of collagen in the pan-specific TGF-β antibody treated group and a trend towards lowered collagen concentration in the TGF-β_2 antibody treated group. These data demonstrated that inhibition of TGF-β was associated with an inhibition of collagen production, which may also have contributed to decreased tumour growth.

7.6 Summary and conclusions

The presence of TGF-β_1 in solid tumours was confirmed by immunohistochemistry and TGF-β_1 was found in abundance in the ECM within AC29 tumours. Inhibition of TGF-β_1 had no effect on tumour growth at any of the time points examined and did not alter tumour collagen content. However, neutralising TGF-β_2, the predominant TGF-β isoform produced by AC29, reduced tumour weight at 15 days, with a trend towards decreased collagen content. Evidence in the literature from other studies suggests that improved immune function may be responsible for the decreased tumour growth observed. Pan-specific neutralisation of all TGF-β isoforms reduced tumour weight at 14 days with an associated lower concentration of collagen in the treated tumours. These observations suggest that TGF-β-induced collagen production, at least in part, stimulated MM tumour growth. The importance of collagen production in MM cell proliferation and tumour growth was previously established in chapters 4 and 5.

In summary, this thesis has clearly demonstrated that both collagen production and TGF-β production, especially the -β_2 isoform, are important for MM growth, and inhibition of either collagen production or pan-specific TGF-β activity can delay MM tumour growth with a resultant decrease in tumour collagen content. The overall results and implications of this thesis are discussed in the following final chapter.

Chapter Eight

General Summary and Future Studies

8.1 General Summary and Conclusions

To date, it is unknown whether the tumour stroma is beneficial for the tumour or represents a host defence mechanism to contain the tumour (Lohr *et al.*, 2001). However, all solid tumours require ECM to grow beyond 1 – 2 mm^2 and tumour growth has been likened to wound healing (Dvorak, 1986). Wound healing involves the formation of a provisional matrix and the emergence of blood capillaries, followed by fibroblasts that synthesise further ECM components. Eventually the vessels and fibroblasts clear and the wound resolves leaving a scar. Tumours appear to be 'unending wounds that do not heal', and perhaps under the guise of a wound evade host rejection.

The first hypothesis of this thesis assessed the importance of collagen, a major ECM component of tumours and wounds, on MM tumour growth:

1) The ECM is vital to the progression of malignant mesothelioma with collagen production stimulating cell proliferation and tumour growth.

It was demonstrated in chapters 3 – 5 that MM tumour cells produced elevated levels of collagen compared to non-transformed mesothelial cells and that exogenous collagen stimulated MM cell proliferation. Inhibition of endogenous collagen production with thiaproline decreased MM cell proliferation dose-dependently. These observations indicated an important role for collagen in MM cell proliferation. This role was examined further by the *in vivo* inhibition of collagen production by thiaproline administration, and resulted in a delay in tumour growth at 10 days. The thiaproline treated tumours were smaller than the control tumours and contained a lower concentration of collagen. A very strong correlation was found between collagen content and tumour weight suggesting the two were closely linked. These novel data confirmed the above hypothesis and demonstrated for the first time that inhibition of MM collagen production could attenuate cell proliferation and tumour growth.

Levels of TGF-β, a key regulator of cell proliferation and collagen production, are also elevated in MM (Fitzpatrick *et al.*, 1994, Maeda *et al.*, 1994). As collagen

synthesis had been shown to be important in MM, the effect of TGF-β-induced collagen production on MM growth was investigated by testing the second thesis hypothesis:

2) Autocrine TGF-β production by MM promotes malignant cell proliferation and tumour growth by enhancing collagen production.

Experiments in chapters 3 and 6 confirmed that MM cells produce highly elevated endogenous levels of TGF-β compared to normal mesothelial cells. Additionally, it was shown that the exogenous application of TGF-β_1 to MM cell cultures highly stimulated collagen production in a dose-responsive fashion, although the same range of concentrations also reduced cell proliferation. These responses to TGF-β may explain the extremely fibrous nature of MM (high collagen production) and the long latency period from initial asbestos exposure to disease presentation (inhibition of cell proliferation).

Characterisation experiments revealed that MM produced elevated levels of TGF-β_2 compared with normal mesothelial cells, although *in vitro* isoform specific effects of exogenous TGF-β on cell proliferation were not observed. No alteration in cell proliferation or collagen production was seen with neutralising antibodies to specific TGF-β isoforms. Interestingly, *in vivo* inhibition of TGF-β_2 resulted in a highly significant decrease in tumour weight at 15 days, which was not seen with TGF-β_1 inhibition, suggesting an important role for TGF-β_2 in MM tumour growth, confirming previous studies (Fitzpatrick *et al.*, 1994, Marzo *et al.*, 1997). However, a similar reduction in tumour weight was observed with pan-specific neutralisation of all TGF-β isoforms at 14 days, albeit not to the same extent. There was a trend towards decreased collagen concentration in the TGF-β_2 antibody treated tumours, and this was also observed in the pan-specific TGF-β antibody treated tumours with a significant decrease compared to the PBS control tumours.

These novel data showed that *in vitro* exogenous TGF-β decreased cell proliferation and that inhibition of endogenous TGF-β did not affect collagen production or proliferation. However, *in vivo* TGF-β inhibition delayed tumour growth and reduced

the collagen concentration of the treated tumours. These results clearly demonstrated the importance of collagen in MM solid tumour growth, and the *in vivo* pan-specific TGF-β neutralising experiments indicated that TGF-β-induced collagen production may play a crucial role in tumour growth.

There is an intertwined relationship between the ECM and TGF-β in tumourigenesis, TGF-β stimulates matrix production (Coker *et al.,* 1997) and the matrix acts as a reservoir sequestering latent TGF-β within the vicinity of the tumour (Boudreau and Bissell, 1998). The ECM and TGF-β have multiple roles in the enhancement of malignant growth (summarised in figure 8.1). It has been demonstrated that ECM components are capable of stimulating tumour cell proliferation, migration, invasion and metastasis (Tobin *et al,* 2001) as well as protecting tumours from chemotherapeutic agents (Sethi *et al.,* 1999). TGF-β suppresses the host immune response (Fitzpatrick *et al.,* 1994) and may also be involved in the suppression of apoptosis (Lei *et al.,* 2002). TGF-β also stimulates angiogenesis (Breier *et al.,* 2002) and the recruitment of ECM-producing stromal cells (Berking *et al.,* 2002). Thus, as figure 8.1 demonstrates, inhibition of both collagen and TGF-β production could be a powerful means of attenuating MM tumour growth.

Collectively, the data in this thesis have demonstrated that inhibition of either collagen or TGF-β reduces MM tumour size with an associated decrease in tumour collagen concentration. This clearly shows that collagen-mediated growth is important in MM tumourigenesis, although figure 8.1 demonstrates that this is only one of several interacting processes promoting malignant growth. The localised nature of MM makes it suitable for antibody and pharmaceutical therapies; the pleural membranes would potentially contain introduced agents in the vicinity of tumour. A multimodal approach combining a collagen inhibitor in association with TGF-β neutralisation may prove highly beneficial in the treatment of this disease.

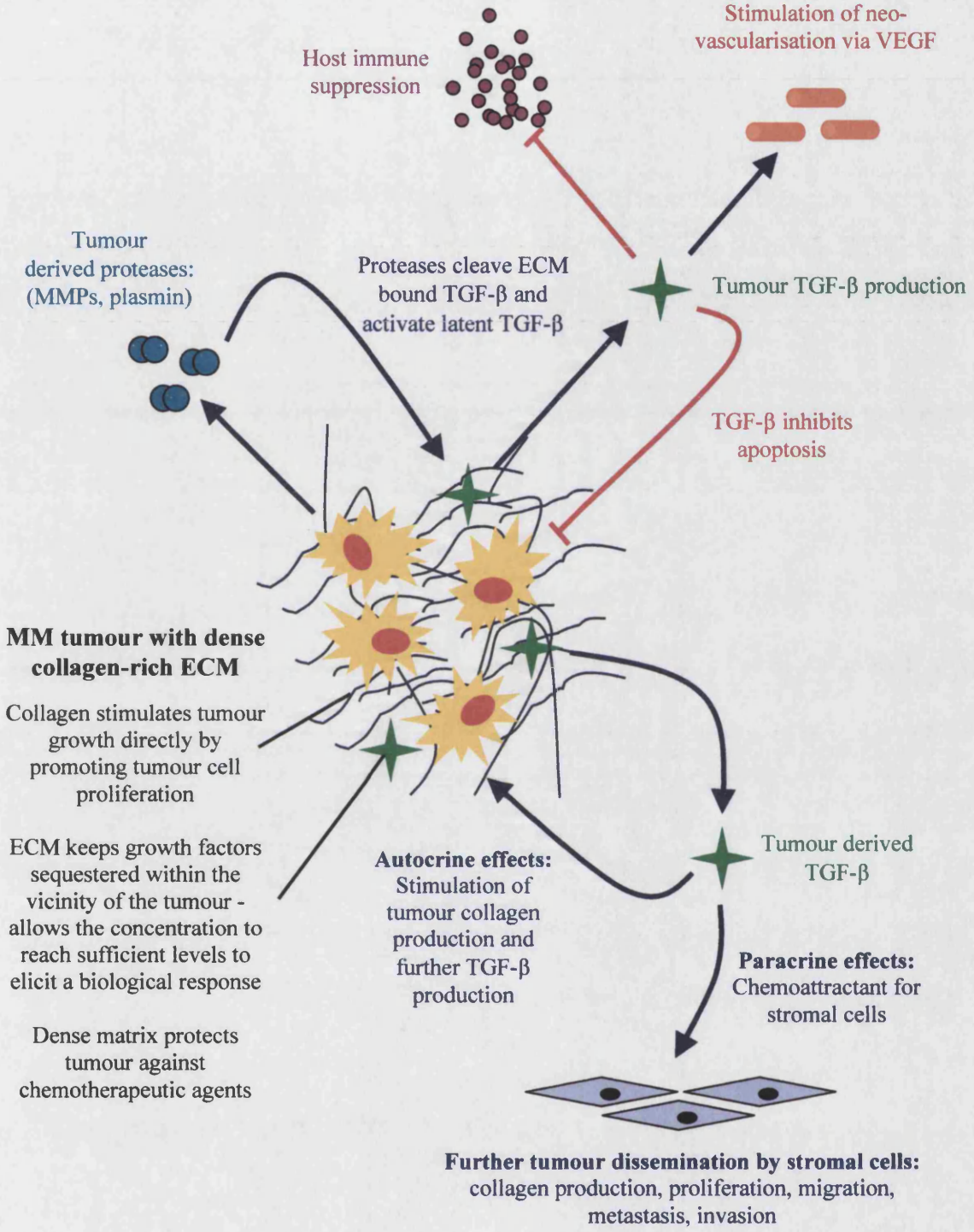


Figure 8.1 Pro-tumourigenic properties of TGF-β and collagen in the MM tumour microenvironment. The interaction of TGF-β and ECM proteins creates a favourable environment in which malignant cells proliferate whilst evading host detection.

8.2 Further studies

To confirm the importance of ECM to tumour growth, inhibition of β_1 integrin, a major cell - ECM attachment molecule, could be achieved with a function blocking antibody and the effect on *in vitro* cell proliferation and *in vivo* tumour growth observed (Sethi *et al,* 1999).

Having determined an important role for TGF-β induced collagen production in MM tumour growth, the contribution of the other possible effects of inhibiting collagen or TGF-β could be investigated in more detail (figure 8.1). The tissue sections generated from the *in vivo* studies could be analysed by immunohistochemistry, vascular development in the murine model compared by staining for endothelial cell markers (Edwards *et al.,* 2001). Differences in the immune response between treated and control tumours could also be assessed histologically by using antibodies specific to inflammatory cells such as B-cells, T-cells and macrophages (Fitzpatrick *et al.,* 1994).

Further *in vitro* studies to determine the effect of collagen and TGF-β on other aspects of tumour growth such as invasion and migration could be performed. Rather than using the cell monolayer, culture models more representative of *in vivo* cell-cell and cell-ECM interactions such as 3D gels and tumour spheroid models could be used to mimic the tumour more accurately (Green *et al.,* 2002).

The combined effect of inhibiting both collagen and TGF-β on MM proliferation and tumour growth could be investigated with a combination of thiaproline and TGF-β antibodies. The importance of TGF-β-induced collagen production in the growth of other cell lines in the panel of MM lines characterised, especially the human cell lines in conjunction with nude mice, could also be assessed. Also, the effect of TGF-β antibodies and / or collagen inhibition on the ability of chemotherapeutic agents to induce MM cell apoptosis could be investigated (Sethi *et al.,* 1999).

Presentations and Publications

The proline analogue thiaproline inhibits malignant mesothelioma cell collagen production in vitro and tumour growth in mice

KS Abayasiriwardana, RJ McAnulty, MK Wood, GJ Laurent, SE Mutsaers

Am. J. Resp. Crit. Care Med. (163) 5 A17 2001

Oral presentation, American Thoracic Society, San Francisco, USA, May 2001

Thiaproline delays malignant mesothelioma growth in mice

KS Abayasiriwardana, MK Wood, GJ Laurent, RJ McAnulty, SE Mutsaers

Oral presentation, British Association for Lung Research, Leuven, Belgium, September 2001

Tumour enhancing effects of TGF-β in malignant mesothelioma are not inhibited by blocking the SMAD pathway

MK Wood, **KS Abayasiriwardana**, W Low, GJ Laurent, RJ McAnulty, SE Mutsaers

Poster presentation, British Association for Lung Research, Leuven, Belgium, September 2001

The role of the extracellular matrix in malignant mesothelioma growth

KS Abayasiriwardana, MK Wood, GJ Laurent, RJ McAnulty, SE Mutsaers

Oral presentation, Australian Thoracic Society, Perth, Australia, December 2001

Inhibition of malignant mesothelioma cell collagen synthesis delays tumour growth

KS Abayasiriwardana, MK Wood, GJ Laurent, RJ McAnulty, SE Mutsaers

Thorax (56) S17, 2001

Oral presentation, British Thoracic Society, London, England, December 2001

Tumour enhancing effects of TGF-β in malignant mesothelioma are not inhibited by blocking the SMAD pathway

MK Wood, **KS Abayasiriwardana**, W Low, GJ Laurent, RJ McAnulty, SE Mutsaers

Thorax (56) S18, 2001

Oral presentation, British Thoracic Society, London, England, December 2001

Neutralising antibody to transforming growth factor beta-2 inhibits malignant mesothelioma cell proliferation and tumour growth

KS Abayasiriwardana, MK Wood, GJ Laurent, SE Mutsaers, RJ McAnulty

Am. J. Resp. Crit. Care Med. (165) 8 A206 2002

Poster discussion, American Thoracic Society, Atlanta, USA, May 2002

TGF-β induced collagen production in malignant mesothelioma cells is not reduced by over-expression of inhibitory SMAD7 or by inhibition of ERK1/2

MK Wood, **KS Abayasiriwardana**, GJ Laurent, W Low, MK Collins, RJ McAnulty, SE Mutsaers

Am. J. Resp. Crit. Care Med. (165) 8 A428 2002

Poster discussion, American Thoracic Society, Atlanta, USA, May 2002

Transforming Growth Factor Beta (TGF-β) induces mitogen activated protein kinase (MAPK) signalling pathways in malignant mesothelioma

MK Wood, **KS Abayasiriwardana**, GJ Laurent, SE Mutsaers and RJ McAnulty

Thorax (57) S115, 2002

Oral presentation, British Thoracic Society, London, England, December 2002

Inhibition of Transforming Growth Factor-β_2 activity attenuates mesothelioma tumour growth

KS Abayasiriwardana, MK Wood, GJ Laurent, SE Mutsaers, RJ McAnulty

Oral presentation, International Mesothelioma Interest Group, Perth, Australia, December 2002

Transforming Growth Factor-β regulates malignant mesothelioma collagen production and tumour growth

MK Wood, **KS Abayasiriwardana**, GJ Laurent, SE Mutsaers, RJ McAnulty

Am. J. Resp. Crit. Care Med. (167) 7 A898 2003

Poster, American Thoracic Society, Seattle, USA, May 2003

Inhibition of Transforming Growth Factor-β_2 activity reduces malignant mesothelioma tumour growth

KS Abayasiriwardana, MK Wood, GJ Laurent, SE Mutsaers, RJ McAnulty

Am. J. Resp. Crit. Care Med. (167) 7 A898 2003

Poster, American Thoracic Society, Seattle, USA, May 2003

Inhibition of collagen production reduces murine mesothelioma cell proliferation and delays tumour growth

KS Abayasiriwardana, MK Wood, GJ Laurent, BW Robinson, RJ McAnulty, SE Mutsaers

Manuscript submitted to J. Pathology, July 2003

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